*Volume 50* *August 1963* *Number 2*

# *Proceedings of the*
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THE PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
*is published monthly by*
THE NATIONAL ACADEMY OF SCIENCES

*Proceedings of the*
NATIONAL ACADEMY OF SCIENCES

*Volume 50 • Number 2 • August 15, 1963*

# *THE DRUG-REQUIRING PHASE IN THE GROWTH OF DRUG-DEPENDENT ENTEROVIRUSES**

By Hans J. Eggers, Edward Reich, and Igor Tamm

THE ROCKEFELLER INSTITUTE

*Communicated by Frank L. Horsfall, Jr., May 23, 1963*

It has been shown recently that the intriguing phenomenon of drug dependence occurs not only in bacteria, but also in viruses.[1-4] Variants of enteroviruses have been isolated which require for their multiplication 2-($\alpha$-hydroxybenzyl)-benzimidazole (HBB) or guanidine, two chemical compounds which specifically inhibit the reproduction of members of this group of viruses.[5-11] Dependence is a property of the genetic material of the virus: infective RNA extracted from drug-dependent virus requires the drug to induce production of dependent virus.[1, 10]

It should be emphasized that the single cycle growth characteristics of drug-dependent virus growing in the presence of the drug at optimal concentration are similar to those of the drug-sensitive parent virus growing in the absence of the compound.[10] Furthermore, the drug concentrations required for maximal growth of dependent mutants are strikingly similar to those causing marked inhibition of sensitive parent viruses.[10] These results suggest that dependence and sensitivity involve opposite drug effects at the same site of action. Some evidence in support of this view was obtained in experiments on the effects of HBB and guanidine on the production of poliovirus-induced RNA polymerase.[12] The compounds prevented the appearance of virus-induced RNA polymerase activity in cells infected with drug-sensitive virus; but, on the other hand, guanidine was required for the appearance of this enzyme activity in cells infected with guanidine-dependent virus.

The kinetic and biochemical experiments reported in the present communication show that the HBB- or guanidine-requiring phase in the growth of dependent virus corresponds to the drug-sensitive phase in the growth of sensitive virus; the compounds are required for the replication of the RNA of dependent virus, whereas they prevent the replication of the RNA of sensitive virus.

*Materials and Methods.*—Coxsackie A9 virus (Woods strain) and its HBB-dependent variant were grown in primary cultures of rhesus monkey kidney cells;[10, 13] poliovirus (Brunhilde strain) and its guanidine-dependent variant, kindly made available by Dr. Nada Ledinko, The Public Health Research Laboratories of The City of New York, were grown in HeLa cells in suspension.[4, 12] Infective virus

was assayed by the plaque technique in monkey kidney cell cultures.[14] The HBB-dependent Coxsackie A9 virus was assayed in the presence of 22 $\mu$g/ml HBB, and the guanidine-dependent poliovirus in the presence of 100 $\mu$g/ml guanidine hydrochloride. All growth curve experiments were carried out at 37°C in a constant temperature room.

Growth curve experiments with Coxsackie A9 virus were carried out as described previously.[13] Synthesis of viral RNA was measured by extraction and assay of infective viral RNA or by incorporation of uridine-$H^3$ into RNA of actinomycin-treated cells.[13, 15]

Experiments with poliovirus in HeLa cells were performed as follows: HeLa cells, $10^6$ cells/ml, were suspended in serum-free Eagle's spinner medium[16] with 4 mM glutamine, and incubated for $1^1/_2$ hr in the presence of actinomycin D (5 $\mu$g/ml). The cells were sedimented in the centrifuge and infected in a small volume ($8 \times 10^7$ cells in 2 ml) with concentrated poliovirus at a multiplicity of 20 infective units of virus per cell. After 15 min adsorption at room temperature, the cells were washed once, resuspended to the original density in warm spinner medium containing 5 $\mu$g/ml of actinomycin D and 1 $\mu$c/ml of uridine-$H^3$ (specific activity 0.5 $\mu$c/$\mu$g), and incubation at 37°C was begun (zero time). At intervals, 10 ml samples were chilled in $N/4$ perchloric acid for determination of uridine incorporation into RNA,[13] and simultaneously 0.5 ml samples were frozen in an alcohol-dry ice bath for determination of virus infectivity. For addition or removal of compound, aliquots of cell suspension were centrifuged in the constant temperature room at 37°C and resuspended in the desired medium.

2-($\alpha$-Hydroxybenzyl)-benzimidazole was obtained through the kindness of Dr. Karl Folkers of Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

*Results.—The HBB-requiring phase in the growth cycle of HBB-dependent Coxsackie A9 virus:* The time course of the HBB-requiring phase in the multiplication of HBB-dependent mutant virus was compared with that of the HBB-sensitive phase in the reproduction of HBB-sensitive parent virus. First, the time of onset of the critical phase was determined. Figure 1 shows the development of HBB-dependent Coxsackie A9 virus in a single cycle of multiplication when the compound was added 1, 2, 3, or 4 hr after virus inoculation. The latent period was 4 hr in control cultures which contained HBB throughout the multiplication cycle. There followed a rapid increase in virus, and maximum yields were reached 8 hr after virus inoculation. When HBB was added either 1 or 2 hr after virus inoculation, the course of virus production was indistinguishable from that in control cultures. When HBB was added 3 or 4 hr after the virus, the latent period was 5 or $5^1/_2$ hr, thus being extended 1 or $1^1/_2$ hr beyond normal. These experiments demonstrate that HBB is not required during the first half of the latent period; the HBB-requiring phase begins in the second half of the latent period.

Corresponding experiments with the HBB-sensitive parent virus are shown in Figure 2. In this case, HBB was present initially, but was then removed at various times after virus inoculation. The latent period was $3^1/_2$ hr, and maximum yields were reached at about 8 hr in control cultures, which did not contain HBB at any time. There was no substantial difference between the growth of virus in control cultures and the curves obtained when HBB was removed 1 hr or $1^3/_4$ hr after virus

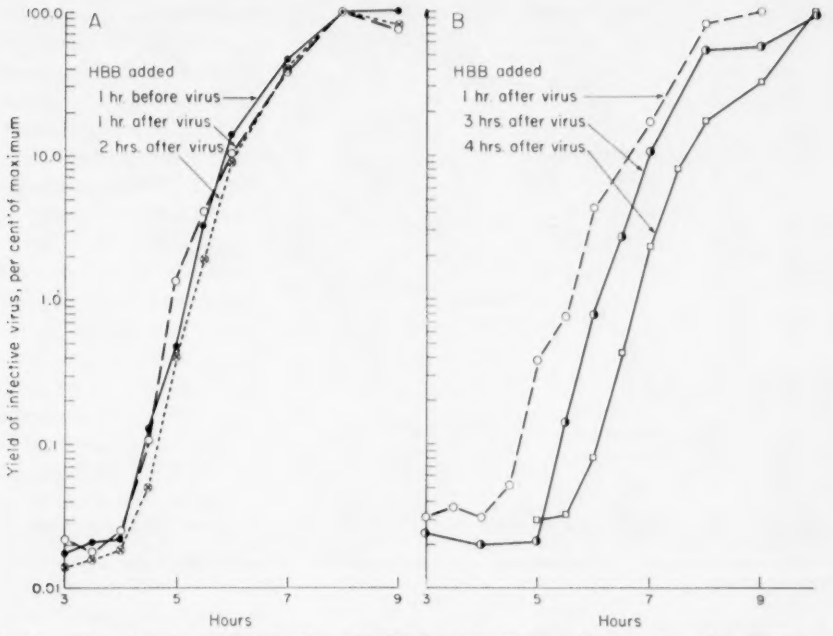


FIG. 1.—Time course of multiplication of HBB-dependent Coxsackie A9 virus when HBB was added 1, 2, 3, or 4 hr following virus inoculation.

inoculation. However, virus reproduction was significantly delayed when cultures were treated with HBB for 3 hr: the latent period was prolonged $1^1/_2$ hr beyond normal. These results show that during the growth cycle of the HBB-sensitive parent virus, the HBB-inhibitable phase begins in the second half of the latent period.

Next, the duration of the HBB-requiring or the HBB-sensitive phase was investigated. In the experiment performed with HBB-dependent virus (Fig. 3, left), HBB was present from the beginning, but was removed $5^1/_2$ hr after virus inoculation, i.e., during the exponential increase in

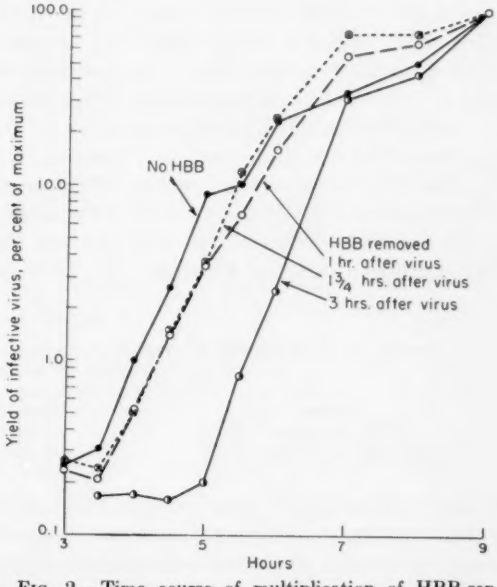


FIG. 2.—Time course of multiplication of HBB-sensitive Coxsackie A9 virus with HBB (49 μg/ml) present for 1, $1^3/_4$, or 3 hr following virus inoculation.

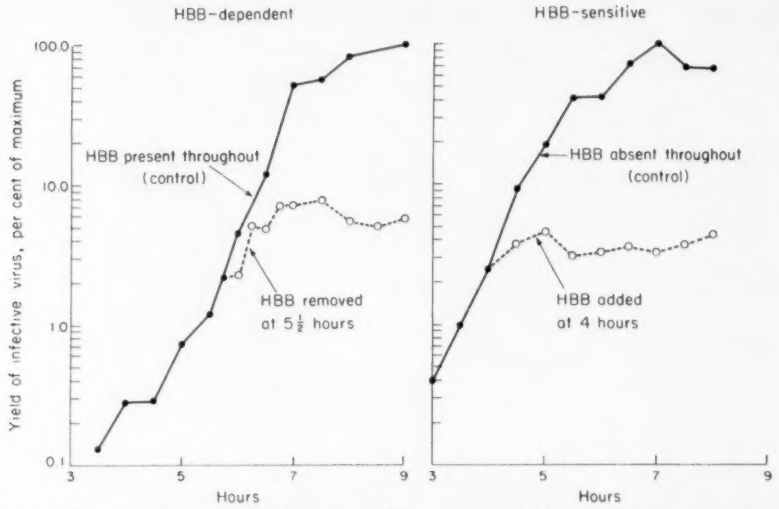


FIG. 3.—Effect of removal or addition of HBB on the multiplication of HBB-dependent or HBB-sensitive Coxsackie A9 virus during the exponential increase phase in virus. HBB-dependent virus was grown in the presence of 22 $\mu$g/ml HBB.[10] 49 $\mu$g/ml HBB was added to the sensitive strain.

virus. Within 30–45 min after removal of compound the production of virus came to a complete halt. Thus, the HBB-requiring phase extends from the beginning of the second half of the latent period well into the exponential increase phase.

Analogous findings were obtained with the HBB-sensitive parent (Fig. 3, right). When HBB was added 4 hr after virus inoculation, virus reproduction stopped within 15 min. A similar result was obtained when HBB was added to the cultures $4^1/_2$ hr after virus inoculation. Thus, as with the HBB-requiring phase in the multiplication of HBB-dependent virus, the HBB-sensitive phase in the reproduction of the drug-sensitive virus also extends from the beginning of the second half of the latent period well into the exponential increase phase.

*Replication of RNA of HBB-dependent Coxsackie A9 virus: Requirement of HBB:* It was reported previously that HBB inhibits the synthesis of the viral RNA of HBB-sensitive Coxsackie A9 virus.[13, 15] Table 1 shows that HBB was required

TABLE 1

EFFECT OF HBB ON THE SYNTHESIS OF INFECTIVE RNA OF HBB-DEPENDENT COXSACKIE A9 VIRUS

| Compound | 8-hr yield, multiple of 2-hr value | |
|---|---|---|
| | RNA | Virus |
| HBB, 22 $\mu$g/ml | 76 | 188 |
| None | 0.93 | 0.7 |

for the synthesis of the infective viral RNA of HBB-dependent virus. Further evidence in support of this conclusion was obtained in experiments on the incorporation of uridine-$H^3$ into actinomycin-treated infected cells under conditions where cellular DNA-dependent RNA synthesis is inhibited more than 90 per cent.[15] Figure 4 shows that infection of actinomycin-treated cells with HBB-dependent

virus caused a marked increase in the uptake of uridine-$H^3$ into acid-insoluble form, provided that HBB was present in the culture. The first significant uptake of label in HBB-treated infected cultures occurred 4–5 hr after virus inoculation, i.e., at the time when virus maturation first becomes demonstrable. This is consistent with previous findings that enterovirus RNA is synthesized at the time of virus assembly, or only a short time earlier.[17, 13, 15]

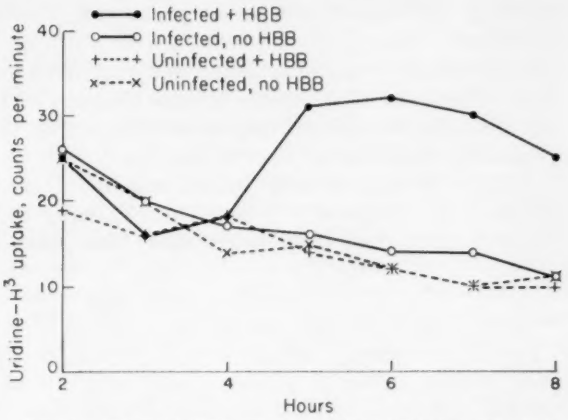


FIG. 4.—Effect of HBB on virus-induced RNA synthesis in actinomycin-treated monkey kidney cells infected with HBB-dependent Coxsackie A9 virus. Actinomycin D (1 $\mu$g/ml) was present 2 hr prior to virus inoculation and throughout the viral multiplication cycle. At intervals, cells were exposed to 1.0 $\mu$c uridine-$H^3$ for 15 min, and uptake into acid-insoluble form was measured.[15]

These experiments demonstrate that the RNA of HBB-dependent virus requires HBB for replication; that of the sensitive virus cannot replicate in the presence of the compound.[13, 15]

The data presented in Figure 4 require one further comment. The uptake of uridine was consistently lower in cells infected with HBB-dependent virus compared with that in cells infected with HBB-sensitive virus. This is probably related to the fact that actinomycin, 1 $\mu$g/ml, reduces the yield of HBB-dependent virus about 4-fold, but does not significantly affect the yield of HBB-sensitive parent virus.

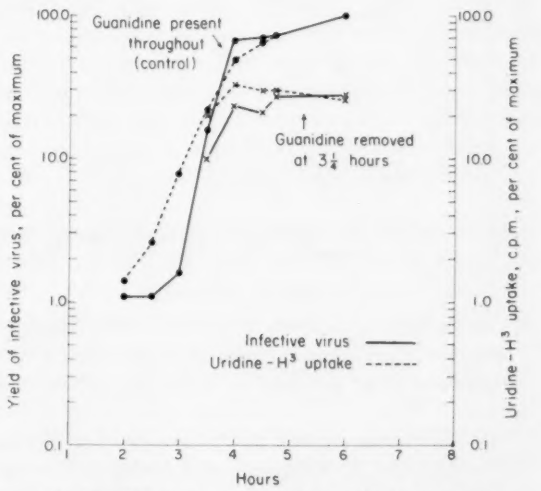
Guanidine present throughout (control)
Guanidine removed at 3¼ hours
Infective virus
Uridine-H³ uptake
Yield of infective virus, per cent of maximum
Uridine-H³ uptake, c.p.m., per cent of maximum
Hours

FIG. 5.—Effect of removal of guanidine on the synthesis of the viral RNA, and on the growth of guanidine-dependent poliovirus 1. Actinomycin D (5 $\mu$g/ml) was present 1½ hr prior to virus inoculation and throughout the viral multiplication cycle. Viral RNA synthesis was measured by incorporation of uridine-$H^3$ into RNA.[13]

*Replication of RNA of guanidine-dependent poliovirus 1: Requirement of guanidine:* The guanidine-dependent mutant of poliovirus 1 and the guanidine-sensitive parent virus were studied in experiments which were in several respects similar to those described for Coxsackie A9 virus and its HBB-dependent variant. Figure 5 records the single cycle growth curve of guanidine-dependent poliovirus, and the

course of synthesis of its RNA as measured by incorporation of uridine-$H^3$. As can be seen, when guanidine was removed at $3^1/_4$ hr, i.e., during the exponential increase phase, virus multiplication and viral RNA synthesis came to a complete halt. These results are similar to those obtained with HBB-dependent Coxsackie A9 virus, but in addition they show directly that the replication of viral RNA requires the continued presence of guanidine.

In an experiment with the guanidine-sensitive parent virus, the compound was added to the cultures at $2^2/_3$ or $3^1/_4$ hr, i.e., during the exponential increase phase. Figure 6 shows that, 15 to 30 min later, virus multiplication and viral RNA syn-

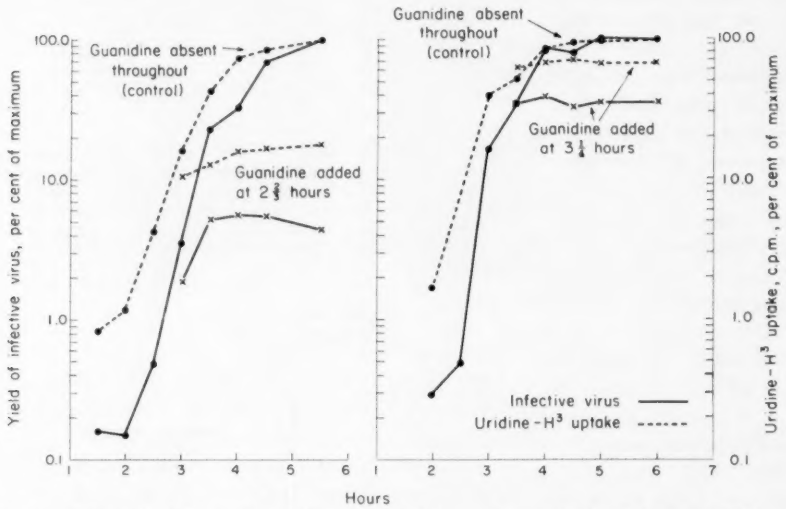


FIG. 6.—Effect of addition of guanidine (150 $\mu$g/ml) on the synthesis of the viral RNA and on the growth of guanidine-sensitive poliovirus 1. Viral RNA synthesis was measured as described in Fig. 5.

thesis became completely inhibited. It is important to note that this occurred even when the compound was added late, i.e., at $3^1/_4$ hr, when about 25 per cent of the total virus and an even larger proportion of the viral RNA had already been formed.

An experiment similar to that described in Figure 6 was carried out with HBB. The compound, at 68 $\mu$g/ml, was added to the cultures at $2^2/_3$ or $3^1/_4$ hr. Like guanidine, HBB inhibited both viral RNA synthesis and virus growth. However, the inhibition was incomplete in that some replication of viral RNA and infective virus occurred. It is known that polioviruses, and particularly some poliovirus 1 strains, are not very sensitive to the action of HBB.[5]

*Discussion.*—We have shown in the present study that the time course of the HBB-requiring phase in the growth cycle of HBB-dependent Coxsackie A9 virus is strikingly similar to that of the HBB-sensitive phase in the reproduction of the HBB-sensitive parent virus. In each case, the critical period begins toward the end of the first half of the latent period and continues well into the exponential increase phase of virus growth. Our results furthermore show that the compound

is necessary for the replication of the RNA of the dependent mutant, whereas it inhibits RNA synthesis of the sensitive parent virus. It thus appears that the critical processes in HBB dependence and HBB sensitivity are functionally and biochemcally analogous. There is evidence from previous work[8, 18] that in the growth cycle of drug-sensitive enteroviruses a guanidine-sensitive process begins very shortly after infection, i.e., 1–2 hr before the onset of the HBB-sensitive phase. In the present study with the guanidine-dependent mutant of poliovirus and its drug-sensitive parent, a kinetic analysis of guanidine requirement or sensitivity during the early phases of viral growth was not carried out. It was shown, however, that the guanidine-requiring or -sensitive phase in the reproductive cycle of poliovirus 1 extends through the exponential increase phase in virus. Furthermore, it was shown that the presence of guanidine during the exponential phase is necessary for RNA synthesis of the dependent mutant, but is inhibitory for RNA synthesis of the sensitive parent virus. These results too are compatible with the hypothesis that the drug-dependent and drug-sensitive processes are biochemically analogous.

Further experimental support for this hypothesis comes from our recent findings concerning the appearance in poliovirus-infected HeLa cells of an RNA polymerase activity not demonstrable in uninfected cells.[12] The appearance of the RNA polymerase activity was inhibited by HBB and guanidine in cells infected with drug-sensitive virus, but required guanidine when cells were infected with the guanidine-requiring mutant. All of the results obtained support the hypothesis that HBB and guanidine have directs effects on the synthesis of virus-induced RNA polymerase, and thereby affect the replication of viral RNA. At the same time they strongly support the inference that the virus-induced RNA polymerase is in fact the enzyme system responsible for the synthesis of viral RNA. The nature of the early process in enterovirus growth, which is sensitive to guanidine but not to HBB, is not clear.

It has been shown with the aid of two inhibitors of protein synthesis, *p*-fluorophenylalanine and puromycin, that formation of poliovirus RNA is dependent on continuing protein synthesis.[19] If virus-induced RNA polymerase is indeed the protein on which the synthesis of viral RNA depends, then it follows that the synthesis of this enzyme continues after completion of the latent period in the multiplication cycle of the virus, and that its functional life is short. These conclusions have been experimentally verified in the case of the virus-induced RNA polymerase demonstrable in poliovirus-infected HeLa cells.[12] The experimental evidence obtained in our laboratory contradicts the notion that the protein on which the synthesis of viral RNA depends is nonenzymatic, as suggested by Wecker.[20]

The genetic analysis of the drug-sensitive, drug-resistant, and drug-dependent states of enteroviruses is as yet too incomplete to permit definite conclusions as to whether these phenotypes are controlled by a single gene occurring in several allelic states. However, the kinetic and biochemical results so far obtained with the mutant strains are compatible with this idea.

We wish to thank Mrs. Helen Algase for excellent technical assistance.

* Aided by a grant from The National Foundation.

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## *THE EINSTEIN GENERALIZED RIEMANNIAN GEOMETRY*

BY LUTHER P. EISENHART

PRINCETON UNIVERSITY

*Communicated May 17, 1963*

1. In the fourth edition of *The Meaning of Relativity*, 1953, Einstein introduced the nonsymmetric covariant metric tensor $g_{ij}$ of the space-time continuum of four dimensions and the contravariant tensor $g^{ij}$ by the equations on page 135

$$g_{ih}g^{ih} = 1,\ g_{hj}g^{hj} = 1,\ g^{ih}g_{il} = 0,\ g_{hj}g^{lj} = 0, \tag{1}$$

where $i$ and $j$ are summed from 1 to 4, $h$ is not summed, and $h$ and $l$ are different.

On page 137 of the fifth edition, he introduces the equation

$$g_{ik,l} - g_{sk}\Gamma_{il}{}^{s} - g_{is}\Gamma_{lk}{}^{s} = 0,$$

which, with change of indexes, is

$$g_{ij,k} = g_{hj}\Gamma_{ik}{}^{h} + g_{ih}\Gamma_{kj}{}^{h}. \tag{2}$$

Each of the terms in the right-hand member of this equation stands for the sum of terms as $h$ takes values 1 to 4. In what follows, when in a term there is the same upper and lower index, the term stands for the sum of four terms as the index takes the values 1 to 4. In the left-hand member of equation (2), $g_{ij,k}$ is the derivative of $g_{ij}$ with respect of $x^k$. This notation is used in what follows.

2. When equation (2) is multiplied by $g^{ih}$ and summed for $i$, in accordance with equation (1), the result is

$$g^{ih}g_{ij,k} = g^{ih}g_{hj}\Gamma_{ik}{}^{h} + \Gamma_{kj}{}^{h},$$

from which,

$$\Gamma_{kj}{}^{h} = g^{ih}g_{ij,k} - g^{ih}g_{mj}\Gamma_{ik}{}^{m}. \tag{3}$$

When equation (2) is multiplied by $g^{hj}$ and summed for $j$ in accordance with equation (1), the result is

$$g^{hj}g_{ij,k} = \Gamma_{ik}{}^{h} + g^{hj}g_{ih}\Gamma_{kj}{}^{h},$$

from which,

$$\Gamma_{ik}{}^{h} = g^{hj}g_{ij,k} - g^{hj}g_{im}\Gamma_{kj}{}^{m}. \tag{4}$$

From this equation,

$$\Gamma_{ik}{}^{m} = g^{mj}g_{ij,k} - g^{mj}g_{it}\Gamma_{kj}{}^{t}.$$

When this expression $\Gamma_{ik}{}^{m}$ is substituted in equation (3), by means of equation (4), the result is

$$\Gamma_{kj}{}^{h} = g^{ih}g_{ij,k} - g^{mj}g_{mj}\,g^{ti}g_{ij,k} + g_{mj}g^{mj}\Gamma_{kj}{}^{h},$$

from which,

$$\Gamma_{kj}{}^{h}(1 - g_{mj}g^{mj}) = g^{ih}g_{ij,k}(1 - g_{mj}g^{mj}).$$

From this equation,

$$\Gamma_{kj}{}^{h} = g^{ih}g_{ij,k}. \tag{5}$$

From (3),

$$\Gamma_{kj}{}^{m} = g^{im}g_{ij,k} - g^{im}g_{ij}\Gamma_{ik}{}^{j}.$$

When this expression for $\Gamma_{kj}{}^{m}$ is introduced in equation (4), the result is

$$\Gamma_{ik}{}^{h} = g^{hj}g_{ij,k} - g^{hj}g_{im}(g^{im}g_{ij,k} - g^{im}g_{ij}\Gamma_{ik}{}^{h}),$$

from which,

$$\Gamma_{ik}{}^{h} = g^{hj}g_{ij,k}(1 - g_{im}g^{im}) + g_{im}g^{im}\Gamma_{ik}{}^{h},$$

from which,

$$\Gamma_{ik}{}^{h}(1 - g_{im}g^{im})g^{hj}g_{ij,k}(1 - g_{im}g^{im}).$$

From this equation,

$$\Gamma_{ik}{}^{h} = g^{hj}g_{ij,k}. \tag{6}$$

3. When $i$ and $j$ are interchanged in equation (2), the resulting equation is

$$g_{ji,k} = g_{hi}\Gamma_{jk}{}^{h} + g_{jh}\Gamma_{ki}{}^{h}. \tag{7}$$

When this equation is multiplied by $g^{hi}$ and summed for $i$, in accordance with equations of the type (1), the result is

$$g^{hi}g_{ji,k} = \Gamma_{jk}{}^{h} + g^{hi}g_{jh}\Gamma_{ki}{}^{h},$$

from which

$$\Gamma_{jk}^{h} = g^{hi}g_{ji,k} - g^{hi}g_{jm}\Gamma_{ki}^{m}. \tag{8}$$

When equation (7) is multiplied by $g^{jh}$ and summed for $j$, the result is

$$g^{jh}g_{ji,k} = g^{jh}g_{hi}\Gamma_{jk,}^{\ h} + \Gamma_{ki}^{h},$$

from which,

$$\Gamma_{ki}^{h} = g^{jh}g_{ji,k} - g^{jh}g_{mi}\Gamma_{jk}^{m}. \tag{9}$$

From this equation,

$$\Gamma_{ki}^{m} = g^{jm}g_{ji,k} - g^{jm}g_{ti}\Gamma_{jk}^{t}.$$

When this expression for $\Gamma_{ki}^{m}$ is substituted in equation (8), the result is

$$\Gamma_{jk}^{h} = g^{hi}g_{ji,k} - g^{hi}g_{jm}(g^{jm}g_{ji,k} - g^{jm}g_{ti}\Gamma_{jk}^{t}),$$

from which

$$\Gamma_{jk}^{h} = (1 - g_{jm}g^{jm})g^{hi}g_{ji,k} + g_{jm}g^{jm}\Gamma_{jk}^{h},$$

from which,

$$\Gamma_{jk}^{h}(1 - g_{jm}g^{jm}) = g^{hi}g_{ji,k}(1 - g_{jm}g^{jm}).$$

From this equation,

$$\Gamma_{jk}^{h} = g^{hi}g_{ji,k}. \tag{10}$$

From equation (8),

$$\Gamma_{jk}^{m} = g^{mi}g_{ji,k} - g^{mi}g_{ji}\Gamma_{ki}^{t}.$$

When this expression for $\Gamma_{jk}^{m}$ is substituted in equation (9), the result is

$$\Gamma_{ki}^{h} = g^{jh}g_{ji,k} - g^{jh}g_{mi}(g^{mi}g_{ji,k} - g^{mi}g_{ji}\Gamma_{ki}^{t}),$$

from which,

$$\Gamma_{ki}^{h}(1 - g_{mi}g^{mi}) = g^{jh}g_{ji,k}(1 - g_{mi}g^{mi}).$$

From this equation,

$$\Gamma_{ki}^{h} = g^{hj}g_{ji,k}. \tag{11}$$

4. When equation (2) is differentiated with respect to $x^{l}$, the resulting equation is

$$g_{ij,kl} = g_{hj}\Gamma_{ik}^{h}{}_{,l} + g_{ih}\Gamma_{kj}^{h}{}_{,l} + \Gamma_{ik}^{h}g_{hj,l} + \Gamma_{kj}^{h}g_{ih,l}. \tag{12}$$

In accordance with equation (2),

$$\begin{aligned}
\Gamma_{ik}^{h}g_{hj,l} &= \Gamma_{ik}^{h}(g_{mj}\Gamma_{hl}^{m} + g_{hm}\Gamma_{lj}^{m}),\\
&= \Gamma_{ik}^{m}(g_{hj}\Gamma_{ml}^{h} + g_{mh}\Gamma_{lj}^{h}),\\
&= g_{hj}\Gamma_{ml}^{h}\Gamma_{ik}^{m} + g_{mh}\Gamma_{ik}^{m}\Gamma_{lj}^{h};\\
\Gamma_{kj}^{h}g_{ih,l} &= \Gamma_{kj}^{h}(g_{mh}\Gamma_{il}^{m} + g_{im}\Gamma_{lh}^{m}),\\
&= \Gamma_{kj}^{m}(g_{hm}\Gamma_{il}^{h} + g_{ih}\Gamma_{lm}^{h}),
\end{aligned}$$

$$= g_{hm}\Gamma_{il}{}^{h}\Gamma_{kj}{}^{m} + g_{ih}\Gamma_{lm}{}^{h}\Gamma_{kj}{}^{m}. \tag{13}$$

By means of these equations, equation (12) becomes

$$\begin{aligned} g_{ij,kl} = {} & g_{hj}(\Gamma_{ik,}{}^{h}{}_{l} + \Gamma_{ml}{}^{h}\Gamma_{ik}{}^{m}) \\ & + g_{ih}[\Gamma_{kj,}{}^{h}{}_{l} + \Gamma_{ml}{}^{h}\Gamma_{kj}{}^{m}) \\ & + g_{mh}\Gamma_{ik}{}^{m}\Gamma_{lj}{}^{h} + g_{hm}\Gamma_{il}{}^{h}\Gamma_{kj}{}^{m}. \end{aligned} \tag{14}$$

When $k$ and $l$ are interchanged in this equation, and the resulting equation is subtracted from equation (14), the result is

$$g_{hj}\Gamma_{ikl}{}^{h} + g_{ih}\Gamma_{kjl}{}^{h} = 0, \tag{15}$$

where, by definition,

$$\Gamma_{ikl}{}^{h} = \Gamma_{ik,}{}^{h}{}_{l} - \Gamma_{il,}{}^{h}{}_{k} + \Gamma_{ml}{}^{h}\Gamma_{ik}{}^{m} - \Gamma_{mk}{}^{h}\Gamma_{il}{}^{m}, \tag{16}$$

$$\Gamma_{kjl}{}^{h} = \Gamma_{kj,}{}^{h}{}_{l} - \Gamma_{lj,}{}^{h}{}_{k} + \Gamma_{ml}{}^{h}\Gamma_{kj}{}^{m} - \Gamma_{mk}{}^{h}\Gamma_{lj}{}^{m}. \tag{17}$$

On page 71 of the fifth edition of *The Meaning of Relativity*, there is the Einstein equation (70):

$$g_{\sigma\alpha}\Gamma_{\mu\nu}{}^{\sigma} = {}^{1}/_{2}(g_{\mu\alpha,\nu} + g_{\nu\alpha,\mu} - g_{\mu\nu,\alpha}).$$

For $\sigma$, $\alpha$, $\mu$, $\nu$ replaced by $h$, $j$, $i$, $k$, respectively, this equation becomes

$$g_{hj}\Gamma_{ik}{}^{h} = {}^{1}/_{2}(g_{ij,k} + g_{kj,i} - g_{ik,j}). \tag{18}$$

Equation (2), namely,

$$g_{ij,k} = g_{hj}\Gamma_{ik}{}^{h} + g_{ih}\Gamma_{kj}{}^{h},$$

is satisfied absolutely by equation (4) and by the equation

$$g_{ih}\Gamma_{kj}{}^{h} = {}^{1}/_{2}(g_{ij,k} - g_{kj,i} + g_{ik,j}). \tag{19}$$

The symbol $\Gamma_{ik}{}^{h}$ is a generalization of the Christoffel symbol $\left\{ {}_{i\,k}^{h} \right\}$, which is symmetric in $i$ and $k$; equation (2) is a generalization of the equation

$$g_{ij,k} = g_{ih}\Gamma_{jk}{}^{h} + g_{hj}\Gamma_{ik}{}^{h}$$

so as not to be symmetric in $i$ and $j$, and the tensor $\Gamma_{ikl}{}^{h}$ is a generalization of the Riemann tensor $R_{ikl}{}^{h}$.

# *EVIDENCE FOR A REQUIREMENT FOR PROTEIN SYNTHESIS FOR AUXIN-INDUCED CELL ENLARGEMENT**

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*Communicated June 20, 1963*

The basic problem of how auxin causes cell enlargement in higher plants has been studied for years, but with little success. That the growth in size is made possible by an increasing cell-wall plasticity seems fairly clear, but the metabolic events which intervene remain obscure. There is extensive evidence both for and against a methylation process,[1] which modifies the physical properties of the cell-wall pectin, and there are several phenomena which may or may not be directly related, such as the acceleration of protoplasmic streaming and the increase in respiratory rate. This latter, though in general it shows excellent correlation with growth, varies greatly in magnitude in different experimental objects, but the fact that growth is inhibited by anaerobiosis, by fluoride, CO in darkness, 2,4-dinitrophenol, iodoacetate, and arsenite certainly indicates participation of an oxidative source of energy.[2] However, the fact that cell enlargement is much more sensitive to some of the inhibitors than is respiration rate does suggest that the connection may be only indirect. Recently, Ray and Ruesink[3] demonstrated a lag of several minutes (at 25°C) before auxin increases the growth rate of coleoptile sections. Since the processes that occur during the lag have a $Q_{10}$ of about 2, this bears out the idea that one or more metabolic processes intervene.

Among the processes which have from time to time been implicated in cell enlargement is protein synthesis. In some tissues (e.g., artichoke slices[4]) the formation of total protein has been shown to be greatly promoted by auxin; in others, (e.g., potato slices,[4] and pea stem,[5] soybean hypocotyl,[6] or *Avena* coleoptile sections[7]) there is a much smaller promotion which may not always occur. In the corn coleoptile a brief study of the incorporation of $C^{14}$-leucine appears to show no promotion at all,[8] although the actual elongation in those experiments was rather limited. A possible interpretation of the scanty published data is that auxin may promote synthesis or turnover of a protein which in some tissue comprises only a small part of the total protein content, while in others it makes up a more considerable fraction. This paper presents an examination of such a possibility, and offers evidence that synthesis of some protein is indeed among the processes required for cell enlargement.

Instead of measuring total protein, we have followed incorporation of L-leucine-1-$C^{14}$ into suspendible protein and into a readily centrifuged protein preparation ("heavy fraction") which is considered to be in good part cell wall. Much of the work rests on the use of selective metabolic inhibitors which are known to act on various phases of protein synthesis.

A few such inhibitors have been used earlier. Long ago, Bonner[7] showed that the amino acid analogue canavanine inhibited auxin-induced elongation of coleoptile sections, and his data also show an inhibition exerted by hydroxyproline, an analogue of proline.[9] Later, Schrank found ethionine to have a similar effect.[10] The specificity of canavanine and ethionine is unfortunately limited by the par-

ticipation of arginine and methionine in reactions other than protein synthesis. Chloramphenicol, which specifically inhibits protein synthesis in bacteria,[11] has been applied to plants by several workers, but they either obtained no inhibition[12, 13] or, where there was growth inhibition, did not consider it as evidence for the participation of protein synthesis in growth.[14, 15]

Four relatively specific inhibitors have been used here: (1) chloramphenicol, which inhibits protein synthesis in plant tissue[16, 17] and in cell-free plant preparations[18]; (2) puromycin, which appears to inhibit coupling of amino acids by acting as an analogue of transfer RNA with its attached amino acid;[19] (3) para-fluorophenylalanine, a specific antagonist of phenylalanine, which may act by inhibiting protein synthesis[17, 20] or producing abnormal proteins;[21] and (4) actinomycin D, which seems to inhibit DNA-dependent RNA synthesis by binding on to guanine of DNA.[22]

Preliminary reports of some of these experiments have been made previously.[23]

*Materials and Methods.*—Alaska peas were soaked in water for 6 hr and were germinated between damp paper towels. Forty-eight hr after the start of soaking, at 25°C, the seedlings were transferred to a rack with their roots in water. On the seventh day 20 mm sections were taken from the third internode starting about 1 mm below the hook. The sections were placed directly in test solutions containing $C^{14}$-leucine with or without indole-3-acetic acid (IAA) and/or the inhibitors, but without added nutrients.

Unhusked seeds of *Avena sativa* var. Victory were soaked in water for 4 hr and germinated in vermiculite. The seedlings were exposed to red light for about 12 hr starting about 3 days after the beginning of the soaking. At about 92 hr at 25°C, from the initial soakings, coleoptiles about 3 cm long were cut, the primary leaves were removed, and the coleoptiles were floated on water. After $1^1/_2$–2 hr, 10 mm sections were made, starting 2–3 mm below the tip of the coleoptile, and floated on solutions containing $1 \times 10^{-4}$ *M* penicillin GK, $5 \times 10^{-3}$ *M* potassium phosphate buffer at pH 5.5, and 2% sucrose, with or without 10 mg/l IAA and $C^{14}$-leucine. In all cases the coleoptile sections were incubated on a rotary shaker. The oat and pea seedlings received occasional dim red light throughout their growth. The experiments were carried out under dim red light and at 25°C.

Sprouting tubers of Jerusalem artichokes, *Helianthus tuberosus*, were selected and disks were prepared according to the methods given by Thimann and Loos.[4] The oat and pea sections were incubated in the presence of L-leucine-1-$C^{14}$, specific activity 6.4 mc per mmole, with or without IAA and inhibitors for 5 hr. The solutions contained 0.25 or 0.50 $\mu$c, respectively, of labeled amino acid in 20 ml. For artichokes the experiments were carried out with 10 disks per dish suspended on plastic screens so that the liquid film just touched the disks. The disks were given 24 hr of treatment with or without IAA and then exposed to 0.62 $\mu$c of labeled amino acid in 25 ml for 5 hr along with the treatment solutions. IAA stock solutions of 100 mg per liter were adjusted to pH 5.5 with NaOH. All the sections were washed carefully with distilled water and ground with quartz sand at 3°C with a mortar and pestle. The homogenate was diluted to 7.0 ml with distilled water and centrifuged at about $170 \times g$ for 5 min. The sediment was then washed with water, 0.01 *N* NaOH, and finally with $H_2O$ three times. The supernatant from the first two washings was combined with the original supernatant. The aggregate of the supernatant solutions will be referred to as the "supernatant fraction," and the washed sediment will be called the "heavy fraction." The proteins were precipitated by adding 2 volumes of 20% trichloroacetic acid and purified by successive washing.[24] In parallel experiments, the tissues were extracted with 80% ethanol, and the extracts centrifuged, dried, and counted. All samples were plated and counted on a Nuclear-Chicago automatic changer gas flow counter with an efficiency of about 31%.

*Results.*—(a) *Effect of auxin on the incorporation of $C^{14}$-leucine into protein:* Table 1 shows that in pea stem sections auxin increases the incorporation of $C^{14}$-leucine into protein by about 50 per cent and its uptake into the free amino acid pool

TABLE 1

EFFECT OF AUXIN* ON THE FREE $C^{14}$-LEUCINE CONTENT AND ON INCORPORATION OF $C^{14}$-LEUCINE INTO PROTEIN

| | Without IAA | With IAA | Per cent increase | Expts. |
|---|---|---|---|---|
| Pea stem sections | | | | |
| *Growth*, % elongation in 5 hr | 10.9% | 39.0% | | |
| in 24 hr | 20.1 | 55.0 | | |
| *Activity* in cpm per 20 sections | | | | |
| Supernatant protein | 3,330 | 5,300 | 59 | 9 |
| Heavy fraction protein | 612 | 890 | 41 | 7 |
| 80% ethanol extract | 1,160 | 1,810 | 56 | 6 |
| Oat coleoptile sections | | | | |
| *Growth*, % elongation in 5 hr | 9.5% | 32.9% | | |
| in 24 hr | 21.8 | 122.0 | | |
| *Activity* in cpm per 50 sections | | | | |
| Supernatant protein | 9,140 | 9,220 | 1 | 3 |
| Heavy fraction protein | 562 | 536 | − 5 | 3 |
| 80% ethanol extract | 1,865 | 1,687 | −10 | 4 |
| Artichoke tuber disks | | | | |
| *Growth*, % incr. in fresh weight in 24 hr | 4.7% | 22.0% | | |
| *Activity* in cpm per 10 disks | | | | |
| Supernatant protein | 27,900 | 32,700 | 17 | 5 |
| Heavy fraction protein | 10,650 | 16,090 | 51 | 7 |
| 80% ethanol extract | 12,710 | 5,710 | −55 | 7 |

* In all cases the final concentration of IAA used was 10 mg/liter.

by about the same amount. Since IAA has little effect on the total protein content of pea stem sections, these increases may perhaps be due only to stimulated uptake of the amino acid.[5] In *Avena* coleoptile sections, furthermore, IAA seems to have little or no effect on the uptake of $C^{14}$-leucine or on its incorporation into protein under the conditions of these experiments. These data agree with the earlier observations on corn coleoptiles.[3] In artichoke tuber disks, IAA greatly increases the incorporation of labeled leucine into protein during a 5 hr period, with a concomitant decrease of similar magnitude in the amount of free labeled amino acid in the tissues. This can be interpreted as indicating that auxin increases the synthesis of protein,[4] but probably does not increase the uptake of labeled amino acid as it appears to do in pea stem sections. In general, there seems to be no striking preferential effect of auxin on incorporation of labeled amino acids into the protein of the heavy fraction which contains the cell walls, for the incorporation of $C^{14}$-leucine into protein in this fraction during a 5 hr period is not more auxin-sensitive than that into the supernatant protein.

(b) *Inhibition of auxin-induced growth by chloramphenicol:* From preliminary exploration of a wide range of concentrations of this drug, it was determined that the elongation induced by exogenous auxin in pea stem sections is inhibited about 50 per cent by a concentration of $4 \times 10^{-3}M$. Figure 1*a* presents a series of elongation measurements, and demonstrates that the growth due to exogenous auxin as well as that of controls (which is presumably due mainly to endogenous supplies of auxin) are both inhibited in the same range of concentrations. The inhibition is relatively greater, however, when auxin is supplied. The fact that growth without exogenous auxin is still inhibited shows that the drug cannot be acting by inhibiting auxin uptake. Similar results were obtained when $\alpha$-naphthalene-acetic acid or 2,4-dichlorophenoxyacetic acid were used as auxins.

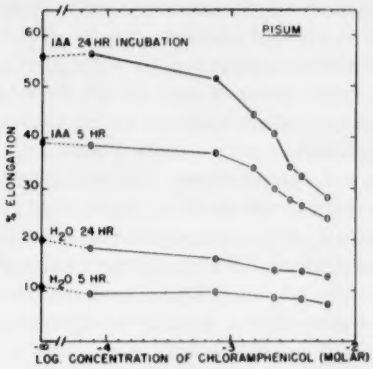


FIG. 1*a*.—Effect of chloramphenicol on the growth of pea stem sections.

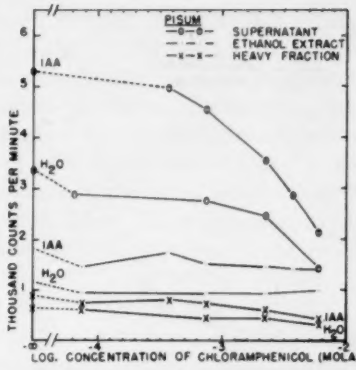


FIG. 1*b*.—Effect of chloramphenicol on the uptake of L-leucine-1-$C^{14}$ and its incorporation into protein in pea stem sections.

*Avena* coleoptile sections behaved much like the pea stem sections of Figure 1, and the data will not be presented here.

(c) *Inhibition of protein synthesis in growing organs by chloramphenicol:* With the above evidence that cell elongation is strongly inhibited by a specific inhibitor of protein synthesis, it was obviously desirable to examine the effect of the same concentrations of chloramphenicol on the uptake and incorporation of $C^{14}$-leucine. As noted above, an inhibiting action of chloramphenicol on protein synthesis has been reported in several plants. Figure 1*b* shows that in fact the very same concentrations which inhibit growth inhibit the incorporation of leucine into protein. There is also a smaller effect on the uptake of leucine, but it is evidently not large enough to account in itself for the effect on protein synthesis. However, the fact that uptake is even partially inhibited might be taken as evidence that a protein or enzyme needs to be formed or turned over in order to bring leucine into the cell.

The inhibition was observed also when the tissues were preincubated in the $C^{14}$-leucine, and subsequently placed in chloramphenicol. Unfortunately, since the leucine begins to be incorporated almost at once, the *relative* inhibition is less under these conditions, but, for example, the supernatant protein of pea stem sections after 4 hr in $6.0 \times 10^{-3}$ *M* chloramphenicol gave 1,670 cpm, while that of controls gave 2,320 cpm, i.e., an inhibition of about 28 per cent. Experiments on *Avena* and artichoke gave similar results. Thus, the inhibition is qualitatively still present although the $C^{14}$-leucine has already been taken up.

In Figure 2 the percentage inhibition of auxin-induced elongation and of the incorporation of $C^{14}$-leucine into protein are plotted together on the same scale. The correlation is striking. Furthermore, both the other types of tissue studied, namely, oat coleoptile sections and artichoke tuber disks, have given very similar correlations.

(d) *The effect of other inhibitors of protein synthesis on auxin-induced growth:* As noted in the introduction, puromycin and actinomycin D both inhibit protein synthesis in microorganisms. Figures 3 and 4 show that both inhibit the elongation of *Avena* coleoptile sections in auxin. In both cases, indeed, the effective concentrations are lower than those of chloramphenicol. With puromycin 50 per cent

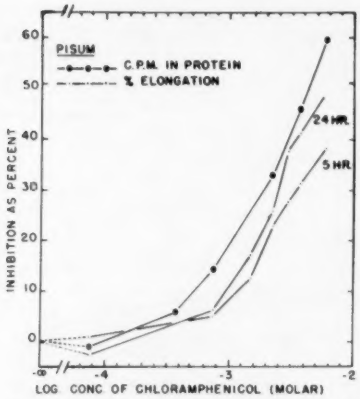


FIG. 2.—Inhibition of growth and protein synthesis in the presence of IAA by chloramphenicol.

inhibition of the auxin-induced elongation is brought about by $2.6 \times 10^{-5}$ *M*, and with actinomycin D by $1.0 \times 10^{-6}$ *M*. Pea stem sections and artichoke tuber disks are also sensitive to puromycin. (Limitations on the supply of pure actinomycin have prevented the repetition of the experiments on these tissues.)

Lastly, D,L-p-fluorophenylalanine has caused strong inhibition of the elongation of *Avena* sections, 50 per cent inhibition of auxin-induced growth being reached in about $1.4 \times 10^{-4}$ *M*.

*Discussion.*—The conclusion that protein synthesis constitutes an essential step in cell enlargement depends, of course, on the specificity of action of the inhibitors used. Chloramphenicol has been shown to inhibit protein synthesis in tissues of carrot root and pea cotyledons,[16, 17] and in a cell-free preparation from *Zea mays*.[18] In these cases, as well as in our own experiments, considerably higher concentrations of chloramphenicol are needed than are effective in bacteria; thus, $6.2 \times 10^{-3}$ *M* inhibited protein synthesis markedly in carrot root slices.[16] That

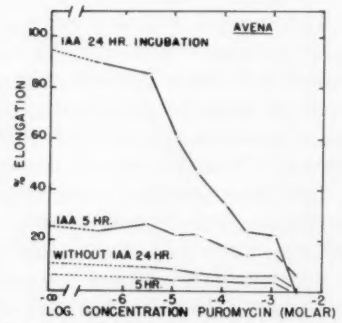


FIG. 3.—Inhibition of the growth of coleoptile sections by puromycin.

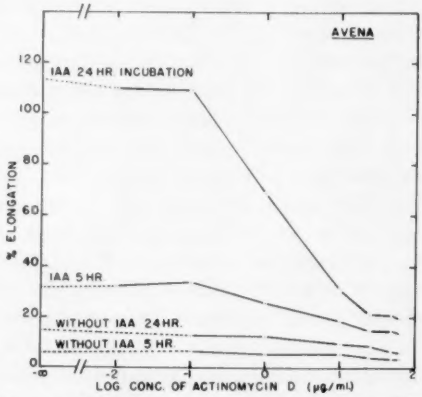


FIG. 4.—Inhibition of the growth of coleoptile sections by actinomycin D.

the action is not due to a generalized toxicity is shown by the fact that concentrations active on protein synthesis have little or no inhibiting effect on respiration in the same tissues[25] or in artichoke tuber disks,[27] although some inhibition of respiration has been reported in wheat root tips.[26] The partial inhibition of leucine uptake shown above, together with this effect on root respiration, suggests that enzymes participating in these two processes may be continually turned over, so that the inhibition would be a secondary one resulting from decreased protein synthesis.

Puromycin inhibits protein synthesis both in bacteria and in animal tissue, and it has been suggested that it acts as an analogue of transfer RNA with its attached amino acid.[19] Since protein synthesis in plants evidently involves a transfer RNA also,[28, 29] the inhibitory mechanism is doubtless the same. Although puromycin is a purine derivative, it appears not to behave as a simple adenine analogue.[19]

In the case of actinomycin D the locus of inhibition in bacteria and in animal tissue is apparently at DNA-dependent syntheses. In isolated pea seedling nuclei it has been shown to inhibit nuclear synthesis of RNA.[29] Thus, its action on protein synthesis may in fact be indirect and mediated through inhibition of the formation of a messenger-type RNA. Such a site of action might well account for the greater effectiveness of actinomycin (mole for mole) than of the other two antibiotics.

Fluorophenylalanine, by displacing or competing with phenylalanine, can either lead to the synthesis of abnormal proteins or block the synthesis. Its effect is thus likely to be specifically directed at protein synthesis proper, rather than at the controlling nucleic acids. It cannot be overlooked, however, that inhibition of protein synthesis may lead to an inhibition of RNA synthesis, or vice versa.

*Summary.*—Cell enlargement is accompanied by considerable incorporation of $C^{14}$-leucine into protein, but, when the enlargement is accelerated by auxin, such incorporation is not accelerated to a comparable degree in all tissues studied. The deduction that synthesis of some one protein, present in differing amounts in different tissues, is a factor limiting growth, has been examined by using antibiotics known to inhibit protein synthesis. Chloramphenicol, puromycin, and p-fluorophenylalanine all inhibit auxin-induced growth, and with the first-named the inhibition of growth and of the incorporation of $C^{14}$-leucine into protein are closely parallel. Auxin-induced growth is also powerfully inhibited by actinomycin D which may indicate a dependence of growth on the synthesis of RNA. The data all indicate that the locus of action of auxin in cell enlargement is on a nucleic acid system controlling synthesis of an essential protein.

* This research was supported in part by a grant to Professor K. V. Thimann from the National Science Foundation, No. G21799, and in part by a U.S. Public Health Service fellowship, No. GF-12,160 to L. D. Noodén from the Division of General Medical Sciences. We wish to thank Lederle Laboratories for puromycin (Stylomycin), Parke, Davis and Co. for chloramphenicol, and Dr. J. O. Lampen for actinomycin D.

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## *HYPERSONIC CHEMOSYNTHESIS AND POSSIBLE FORMATION OF ORGANIC COMPOUNDS FROM IMPACT OF METEORITES ON WATER*

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*Communicated by Henry Eyring, May 29, 1963*

A recently proposed theory[1] of the possible origin of organic compounds on the earth is further developed. The theory is based on the physical and chemical processes associated with the formation of a shock around a large meteorite and subsequent collision of the shock and meteorite with water at hypersonic velocities. Interaction of gaseous species at high temperatures and very high pressures with water molecules should yield a large variety of organic compounds, and a preliminary experiment by W. J. Hooker with a bullet hitting water verified the formation of a large variety of complex organic molecules. A new experiment is suggested and various implications of this theory are discussed. New methods of synthesizing a variety of complex chemical compounds are proposed.

*Previous Theories and Experiments.*—Beutner,[2] Dauvillier,[3] Bernal[4], and Urey[5] suggested the role of lightning in the production of organic compounds in the primitive earth's atmosphere. Experiments with prolonged electric discharges by Miller[6] in Urey's laboratory on a mixture of $CH_4$, $NH_3$, $H_2$, and $H_2O$ vapor produced various amino acids. Later, Abelson[7] obtained similar results with a mixture of $H_2$, $CO_2$ (or CO), $N_2$, and $H_2O$. Pavlovskaya and Pasynskii[8] repeated Miller's experiment and confirmed the formation of amino acids, also obtaining the same results with a mixture of CO, $CH_4$, and $NH_3$ by electric discharges and also by action of ultraviolet radiation. Oparin[9] stated that both ultraviolet radiation and electric discharges played an important role in the formation of organic compounds in the primitive atmosphere. Hayns, Walter, and Meyer[10] also repeated Miller's experiment and extended it to a mixture of $CH_4$, $NH_3$, $H_2O$, and $H_2S$. Lately,[11] other experiments have involved many combinations of gases in the absence of free oxygen and with various other supplies of energy (microwave, ultrasonic, heat,

high energy electrons, Xrays, and proton irradiation).[12–18] In most cases amino acids were found. All of these reactions took place at about atmospheric pressures.

*Present Theory of Hypersonic Organosynthesis.*—A new theory of formation of organic compounds by meteoritic impact on water is further developed in this paper. It should be independent of the detailed composition of the atmosphere, because after passage through a shock the species undergo dissociation and ionization and so lose their original identity, and because the large amounts formed and dissolved in the water would be almost independent of the atmospheric surroundings. Previously, Gilvarry and Hochstim[1] attributed the formation of organic substances by meteoritic impact to quenching of hot species sprayed by water. It now appears likely that three additional factors may have been involved: (1) possible polymerization processes at very high pressures and temperatures created by the impact of the shock wave and meteorite on water and formation of hot gas region; (2) possible catalytic effects of ablating meteorite; and (3) chemical reactions involving meteoritic material.

The theory of hypersonic organosynthesis not only suggests a new source of the necessary energy in the production of various free radicals, but offers multitudes of kinetic paths for their recombination and their collisions with water molecules.

This theory does not preclude the formation of organic compounds by electric discharges, ultraviolet radiation, etc.

*Interaction of Meteorite with Atmosphere.*—First, let us examine what would happen if a large meteorite moved through the earth's atmosphere at hypersonic velocities. In front of the meteorite, which is assumed to be blunt, a detached shock wave would form (see Fig. 1). The region in between would contain com-

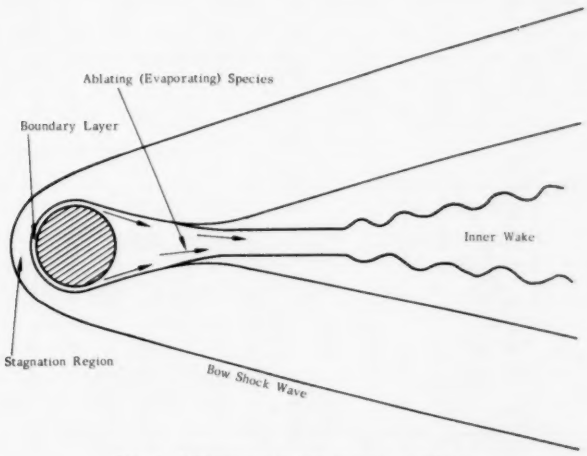


FIG. 1.—Hypersonic flow around a sphere.

pressed gases at very high temperatures. If the primitive atmosphere contained[9] $H_2$, $H_2S$, $CH_4$, $NH_3$, and $H_2O$, these species would, upon crossing the shock wave, become hot enough to dissociate and ionize. For example (see Figs. 2–4), for a meteorite at a velocity of 11 km/sec at sea level in the present composition

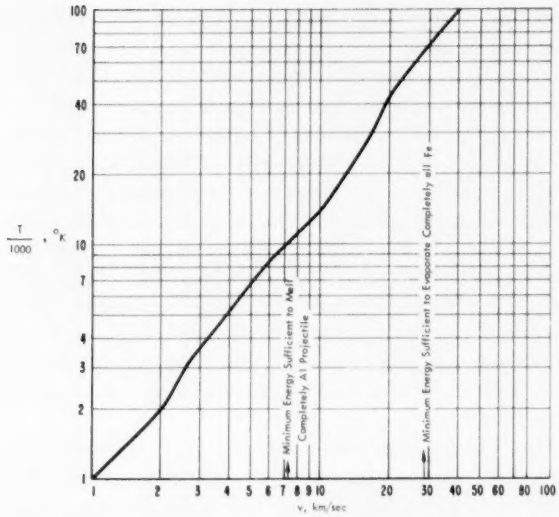


FIG. 2.—Equilibrium temperature in front of projectile versus velocity at sea level in an ideal air (including dissociation and ionization and without radiation losses).

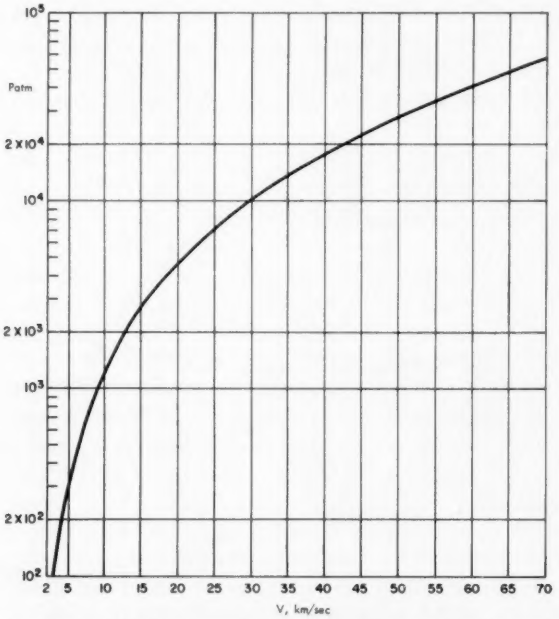


FIG. 3.—Equilibrium pressure in the stagnation region in front of projectile versus velocity at sea level in an ideal air (including dissociation, ionization, and no radiation losses).

of air, the gases in front of a meteorite behind the shock wave (stagnation region) would be at a temperature[19] of 16,300° K, at 11.5 times sea level density, and at a pressure of 1,500 atmospheres (assuming thermodynamic equilibrium, which

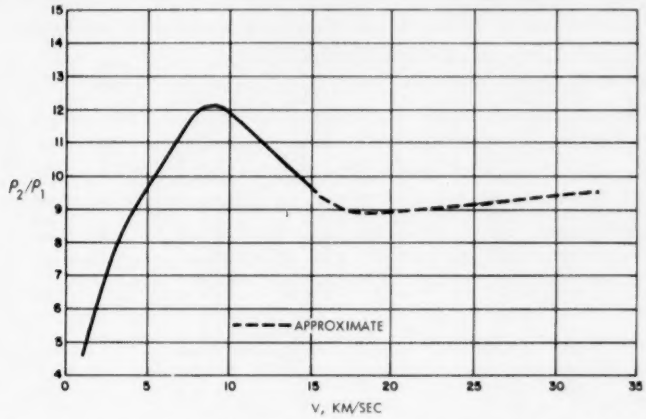


FIG. 4.—Density ratio across the shock in front of projectile in an equilibrium ideal air at sea level (dissociation and ionization included—no radiation).

includes contributions from excitation, dissociation, and ionization). In air or in a primitive atmosphere, in this example, this region would contain[20] N, O, $N^+$, $e^-$, $O^+$, NO, $NO^+$, C, H, $H^+$, OH, etc. At much higher velocities, $N^{++}$, $O^{++}$, $C^{++}$, etc., will become appreciable. These species from the stagnation region flow by the surface of the meteorite, and some portion enters into the boundary layer, which is full of ablated and evaporated meteoritic material. The products of this reaction end in the meteorite's trail (wake). On the bow side of the shock, molecules passing through the shock undergo only partial dissociation and ionization. As the temperature drops behind the meteorite, the various regions will contain:[20] N,O, H, C, $N^+$, $O^+$, $H^+$, $C^+$, $O^-$, $e^-$, $N_2$, $O_2$, $H_2$, $C_2$, NO, OH, CO, CN, CH, NH, SO, HS, $NO^+$, $OH^-$, $O_2^-$, $O_2^+$, $N_2^+$, $H_2^+$, $CH^+$, $CN^+$, $N^{++}$, $O^{++}$, $C^{++}$, $NH_2$, $O_3$, $CH_2$, $CO_2$, $N_2O$, $NO_2$, $H_2O$, HCN, $C_3$, $SO_2$, $H_2S$, $C_2N_2$, $C_4$, $NH_3$, $CH_4$, etc. (and, e.g., from ablation of iron meteorite: Fe, $Fe^+$, FeO, FeN, FeC, Ni, $Ni^+$, CuO, P, PO, etc.). Recombination of these species obeys complicated kinetic rate equations which are coupled with hydrodynamic equations. In the wake, expansion takes place, and some of the species which have slow reaction rates may become chemically frozen. At the far end of the wake, more stable molecules will form, such as $H_2O$, $H_2$, $CO_2$, $O_2$, $N_2$, $SO_2$, $NO_2$, CO, $NH_3$, $CH_4$, CN, $H_2S$, $O_3$, HCN, Fe, FeO, $Fe_2C$, FeOH, FeS, PO, NiO, NiS, etc. The temperatures in the far wake drop off gradually. Thus, the shock transforms the original ambient atmosphere into a completely different one. It is possible that, among the new species formed at the extreme end of the wake, some will be quite complex.[1] From calculations for normal dry air,[20,1] the passage of a meteorite 1 km in diameter would yield in the core of the wake (at $T \simeq 2{,}000°$ K) at least $10^8$ kg of NO, $10^7$ kg of O, $10^6$ kg of $CO_2$, $10^5$ kg of CO, $10^4$ kg of $NO_2$, $10^2$ kg of $N_2O$, etc. At very high velocities of meteorites, radiation from the shocked gases will be sufficiently high in front of the shock to produce preionization and predissociation. As we have pointed out, the hottest region is the stagnation region. The thickness of this region at sea level (see Table 1) for a giant meteorite with a velocity of 11

TABLE 1

SOME PHYSICAL PROPERTIES BEHIND SHOCK WAVES FOR METEORITES AND PROJECTILES IN AIR AT SEA LEVEL

| $R$ | v km/sec | $T_s$ °K | $P_s$, atm | $\rho_s/\rho_1$ | $\frac{d}{R}$ | $m_s$ | $m_{4R}$ | Kinetic Energy, E ergs | Kinetic Energy, E kcal or TNT |
|---|---|---|---|---|---|---|---|---|---|
| 5 mm | 5 | 7,000 | 200 | 9.5 | 0.076 | $4 \times 10^{-4}$ g | $2 \times 10^{-4}$ g | $3.3 \times 10^{11}$ | 7.8 kcal |
| 5 mm | 11 | 16,500 | 1,500 | 11.5 | 0.062 | $4 \times 10^{-4}$ g | $2 \times 10^{-4}$ g | $1.6 \times 10^{12}$ | 38 kcal |
| 1 cm | 5 | 7,000 | 200 | 9.5 | 0.076 | $3 \times 10^{-3}$ g | $1.6 \times 10^{-3}$ g | $2.6 \times 10^{12}$ | 62 kcal |
| 1 cm | 11 | 16,500 | 1,500 | 11.5 | 0.062 | $3 \times 10^{-3}$ g | $1.6 \times 10^{-3}$ g | $1.3 \times 10^{13}$ | 300 kcal |
| 10 cm | 5 | 7,000 | 200 | 9.5 | 0.076 | 3 g | 1.6 g | $2.6 \times 10^{15}$ | 29 g TNT |
| 10 cm | 11 | 16,500 | 1,500 | 11.5 | 0.062 | 3 g | 1.6 g | $1.3 \times 10^{16}$ | 0.32 lb TNT |
| 1 m | 11 | 16,500 | 1,500 | 11.5 | 0.062 | 3 kg | 1.6 kg | $1.3 \times 10^{19}$ | 317 lb TNT |
| 1 m | 30 | 70,000 | 10,000 | ≲9.5 | ≲0.076 | 3 kg | 1.6 kg | $9.4 \times 10^{19}$ | 2.4 KT (TNT) |
| 10 m | 11 | 16,500 | 1,500 | 11.5 | 0.062 | 3 ton | 1.6 tons | $1.3 \times 10^{22}$ | 317 KT |
| 10 m | 30 | 70,000 | 10,000 | ≲9.5 | ≲0.076 | 3 ton | 1.6 tons | $9.4 \times 10^{22}$ | 2.4MT (TNT) |
| 100 m | 11 | 16,500 | 1,500 | 11.5 | 0.062 | $3 \times 10^3$ tons | $1.6 \times 10^3$ tons | $1.3 \times 10^{25}$ | 317 MT |
| 100 m | 30 | 70,000 | 10,000 | ≲9.5 | ≲0.076 | $3 \times 10^3$ tons | $1.6 \times 10^3$ tons | $9.4 \times 10^{25}$ | $2.4 \times 10^3$ MT |
| 500 m | 11 | 16,500 | 1,500 | 11.5 | 0.062 | $4 \times 10^5$ tons | $2 \times 10^5$ tons | $1.6 \times 10^{27}$ | $4 \times 10^4$ MT |
| 500 m | 30 | 70,000 | 10,000 | ≲9.5 | ≲0.076 | $4 \times 10^5$ tons | $2 \times 10^5$ tons | $1.2 \times 10^{28}$ | $3 \times 10^5$ MT |
| 1.5 km | 30 | 70,000 | 10,000 | ≲9.5 | ≲0.076 | $10^7$ tons | $5.4 \times 10^6$ tons | $3.2 \times 10^{29}$ | $8 \times 10^6$ MT |

$R$ = radius of meteorite or projectile before impact; v = velocity of meteorite or projectile on impact; $T_s$, $P_s$ = stagnation temperature and pressure behind shock wave, respectively (in air);[20] $\rho_s/\rho_1$ = density ratio across shock (near stagnation region) in air;[20] $d$ = detachment distance from the shock wave to the surface of the projectile;[21] $m_s \backsimeq 2\rho_1 \left(\frac{\rho_s}{\rho_1}\right)\left(\frac{d}{R}\right) \pi R^3$ = approximate mass of air in stagnation region; approximate mass of air in the wake (2 diameters behind), $m_{4R} \backsimeq 4R \cdot \pi R^2 \ (\rho_1/10)$; $E = {}^1/_2 \left(\frac{4}{3} \pi R^3 \rho_m\right) v^2$, where the density of meteorite $\rho_m$ was used as 5 g/cm³.

km/sec and having 500 meters in radius will be about 30 meters,[21] and gases in this region will weigh about $10^5$ metric tons.

*Collision of Shock Wave and Meteorite with Water.*—Before the shock reaches the water, the water will be heated from the large fluxes of the incident radiation. For our previous example, at T = 16,300° K from the shock, which is radiating almost as a black body, the flux is equal to about 90 kcal/cm²sec, which could evaporate 1 km² area of water at a rate of up to 1 mm/m sec. If the shock collides with water, the water molecules will pass through the shock, partially dissociate, mix, and react at high pressure region in front of the meteorite. These gases in this subsonic region move (in our example), with respect to the surface of the meteorite, with about one-tenth velocity of the meteorite and may have no time to escape around the meteorite, especially if they are captured in the surface cavities and are squeezed into the water.

Meanwhile, the shock which had hit the water's surface is reflected from it (at a velocity[22] of about 3 km/sec), compressing and heating part of the gases rich in hydrogen and oxygen from the water. When the meteorite reaches the original level of the water, the compressed gases may be squeezed into the water (in our example, they weighed $10^5$ metric tons), forming a high pressure and high temperature region which by reacting violently at the interface may form complex compounds. In this stage polymerization reactions could take place. This is also a completely new reaction regime (high pressure, two phase) not simulated previously by Miller and others.[6, 7, 9, 12–16] The meteorite, due to sudden deceleration, explodes (in our previous example, it has kinetic energy equal to 400 hydrogen bombs, each equal to 100 megatons; see Table 1). Meteoritic debris are expelled into the atmosphere and explosion forces a column of water, spray, and

steam up[1, 23] (in our example, the energy is not sufficient to evaporate the meteorite; see Fig. 2).

*Collision of Sprayed Water with Incoming Wake.*—In the meantime, the long and hot wake ($T \gtrsim 2{,}000°$ K) which is coming down with almost the meteorite's velocity collides with the steam and spray moving up.[1] The chemical reactions at this stage should resemble reactions taking place in the collision of a plasma jet with the jet of water vapor.[1] The wake contains large amounts of ablated meteoric species. In an iron meteorite, these are: Fe, Ni, and small amounts of Co, Cu, P, S, C, etc.; in a stony meteorite, $SiO_2$, MgO, FeO, $Al_2O_3$, CaO, $Na_2O$, $K_2O$, etc. Very few meteorites (carbonaceous chondrites) are rich in carbon, hydrogen, nitrogen, oxygen, sulfur, and chlorine.[24, 25] Organic compounds should have been formed by molecular collisions and by quenching of formed species with the water molecules.[1] Slight amounts of sulfur in the ablating meteorite (about 0.04% is found in iron meteorites) or from $H_2S$ or $SO_2$ in the atmosphere may have contributed to the formation of amino acids—cysteine and cystine. The catalytic importance of Fe, Ca, Co, Cu, and K in biological reactions is well known.[35]

*Collision of Bow Shock with Water Surface.*—The shock hits the water with highest velocity near the meteorite surface and with lower velocity as the distance from the center of the impact area increases. Thus, the composition, temperature, and pressure of the interaction region reacting with water changes with distance from the center of impact. Thus, the original bow shock is moving sideways, colliding at the lower part with water suface. It is followed by another shock wave from the exploding meteorite. These shocks at the water interphase could also create organic compounds.

The water which collided with the descending wake would have various complex compounds dissolved in it.[1] Rain of this condensed water containing organic compounds into the oceans, lakes, rivers, and on the land could have provided high local concentrations of organic compounds in the past.

Organic compounds could have formed droplets (coacervates) which did not mix with water.[9, 26] These compounds, in the absence of any life, could have accumulated and reacted with each other until life started in some way which cannot be explained by this theory alone.

*Experiment with Hypersonic Bullet.*—We have proposed two experiments to test this theory.[1] In the first one we suggested firing a ballistic bullet from a hypersonic gun. This bullet, passing through a desired gas composition, was supposed to hit the water with hypersonic velocity.

Dr. W. J. Hooker of General Dynamics, Astronautics in San Diego, California, has built such a gas-water section with thin aluminum membranes and has fired a 6 mm diameter aluminum bullet at a velocity of 4.6 km/sec through an assumed primitive atmosphere (40% $CH_4$, 40% $NH_3$, and 20% $H_2$) in the Hypervelocity Ballistic Range at the Ames Research Center of the National Aeronautics and Space Agency in Mountain View, California. According to the preliminary chemical analysis (by ultraviolet absorption) by Dr. C. Ponnamperuma of the Ames Research Center, the water contained organic compounds.[1, 27] Preliminary chromatographic measurements identified organic compounds of molecular weight up to 200.[27] Further results are to be published by Hooker and by Ponnamperuma.

*Suggestion for New Experiment.*—The previously suggested[1] firing of a hypersonic bullet at $v \gtrsim 2$ km/sec has the limitation that it may not produce significant hot gas bubble and thus possible polymerization processes at high pressures. The bullet, however, sprays the water and produces a quenching and chemical reaction of water with the wake. One could fire a bullet with a U-shaped cavity in the front portion, or one could fire a piston at very high velocities on a mixture of gases. The tube in which the piston can move should be submerged at the end in the liquid. In this way one could form the high temperature gas bubble and hope to detect more complex compounds.

*New Implications from the Theory of Hypersonic Chemosynthesis.*—This theory suggests that complex chemical compounds could be formed by meteoritic impact on any planet which contains any type of atmosphere and liquid phase.

An interplanetary vehicle sent to a planet on a crash landing may, on impact, produce complex chemical compounds which need to be discounted when searching for a sign of life afterwards in the vicinity of the impact.

A new method of synthetically producing a large class of complex chemical compounds can now be proposed. It involves firing, through a gas phase into a liquid phase, projectiles at various velocities and made of various materials. These materials can be made to ablate and chemically react with products of liquid and gaseous phases, or to act catalytically in the polymerization processes. Similar to our previous suggestion,[1] a plasma jet of various composition may produce complex compounds after collision with a liquid phase. As proposed in a previous paragraph, firing of a piston on a gas-liquid interphase should produce high pressure, high temperature polymerization reactions.

*Appendix I. Meteoritic Energies and Large and Giant Meteorites.*—Giant-size meteorites carry tremendous kinetic energy. Thus, a giant meteorite of 1 km in diameter, moving with a velocity of 11 km/sec, has energy equivalent to about $1.6 \times 10^{27}$ ergs $= 4 \times 10^4$ megatons (400 hydrogen bombs, each equal to 100 megatons, or a total of $4 \times 10^{10}$ tons of TNT). This energy is sufficient to evaporate 60 km$^2$ area of ocean, 1 km deep, if all of it is converted only to the evaporization of water. Actually, some of the energy will go to heating and fragmentation of the meteorite, formation of shock waves in water, etc. Many incident meteorites can have original energies up to about 50 times larger. Such meteorites have enough energy to evaporate themselves completely on impact (see Fig. 2).

Large meteorites were highly probable. The Barringer Meteor Crater in Arizona, with a diameter of 1.2 km and depth of 170 m, indicates that the meteorite on impact could have a diameter between 5 and 30 meters.[28] The previously cited example of a 1 km meteorite, if impacted on the earth, would have formed a crater of 40 km in diameter, according to the formula of Innes.[29] The Vredefort Ring in South Africa is of probable meteoritic origin resulting from an impact of a gigantic asteroid about a mile in diameter.[30] Daly[31] and Dietz[30, 31] estimate the real diameter of this crater to be about 50 km. According to Dietz[30] the energy of this asteroid was at least $1.5 \times 10^6$ megatons (35 times more than in our previous example). Recently, Dietz[32, 33] examined the Sudbury lopolith in Ontario and concluded that it probably resulted from a giant meteorite impacting with an energy of about $3 \times 10^{29}$ ergs, which is about 200 times greater energy than we took for our previous example. An asteroid of the size of Juno (diameter about 190 km) would have produced a crater of about 3,300 km in diameter.[34] How many large meteorites hit the water we do not know. However, Dietz[30] and Gallant[34] estimated that 30,000–50,000 large meteorities may have struck the earth during the past 500 million years. This would give[34] roughly on the average one impact every $130 \times 130$ km.[2]

* Guest worker.

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## *MECHANISM FOR SELF-DIFFUSION IN ICE*

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*Communicated June 10, 1963*

The observation that the coefficients of diffusion of deuterium and oxygen-18 in ice are equal strongly suggests that the diffusion measured is the self-diffusion of intact water molecules.[1] Summarized here are the considerations which have led to the conclusion that the diffusion occurs by means of the migration of interstitial molecules.

With ionic solids, the exact relationship between the diffusion constants and the coupled low-frequency conductivity can be valuable in deciding between several conceivable mechanisms.[2] Except for very scarce hydronium and hydroxyl ions,[3] ice is a molecular crystal; it has been proposed by Haas,[4] however, that diffusion in ice is coupled to the displacement current associated with dielectric relaxation. For this to be true, diffusive motion must be accompanied by the displacement of polarization charges; otherwise, as Gränicher[5] has shown, diffusion and dielectric relaxation must be controlled by separate processes.

What are these polarization charges in ice? The structure of ice has been extensively studied;[6] each water molecule forms four hydrogen bonds with its tetrahedrally arranged neighbors. According to Bjerrum,[7] the reorientations of the dipoles associated with dielectric relaxation occur at sites of imperfect hydrogen bond coordination. The defect bonds, containing two protons or none, move as the molecules rotate and are the sources and sinks of the polarization field.[8]

The migration of these "Bjerrum faults" produces no mass transport. However, the activation energy associated with the diffusion process[9] (13.5 ± 1.1 kcal/mole) is very similar to that of dielectric relaxation[10] (13.25 kcal/mole) and led Haas[4] to propose for a common mechanism the migration of pairs formed by the binding of interstitial molecules to the polarization charges. Two models were offered, differing in the motion of the complex: an interstitial might be carried along with a Bjerrum fault for several steps (model A); or at each step the interstitial might displace a lattice molecule as the orientational defect is propagated (model B). Another conceivable electrically active defect, whose motion would produce mass transport, is a Bjerrum fault "diluted" by a vacancy; this imperfection can be imagined to result from the removal of one of the two molecules involved in a defect bond.

It should be remarked that in pure ice the drift of the hydronium and hydroxyl ions, while determining the dc conductivity, contributes little to dielectric relaxation[8] and nothing to oxygen diffusion.

In principle, the importance of the roles played by the various defects could be determined by calculating energies and entropies appropriate to the formation and displacement of the defects. A more feasible approach, however, seems to be an inductive one, based on the experimentally observable consequences of an assumed mechanism.

Experimentally,[10] the dielectric properties of ice can be described by a single relaxation time $\tau_d$—the characteristic time of the exponential decay of the polarization following a discontinuous change in impressed voltage. Consistently, dipole rotation by the drift of orientational defects leads to a prediction of a single relaxation time.[8, 11] Furthermore, if composite defects were predominant, the dielectric relaxation time would be closely related to the coefficient of self-diffusion $D$; this relationship is determined by geometry alone, provided the defect concentration is not extremely high or low.

In calculating the diffusion coefficient or dielectric relaxation time for an assumed mechanism, care must be exercised to account for the various correlation effects between successive jumps. Analyses of the composite defect mechanisms leads to the results contained in the following table,[11] where $d$ is the oxygen-oxygen distance, 2.76 A.

COMPARISON OF DIFFUSION AND DIELECTRIC RELAXATION

| | Model A | Model B | Vacancy-Bjerrum fault pair | Observed |
|---|---|---|---|---|
| $\frac{6D\tau_d}{d^2}$ | 2 | <2 | <1 | $20 \pm 4$ |

The observed diffusion is an order of magnitude faster than dielectric relaxation, if the experiments are discussed in equivalent terms. It can therefore be concluded that the imperfections relevant to diffusion are not, on the whole, electrically active. It is still possible for some, or even most, of the orientational defects to be transport active, but most of the diffusion must be due to another mechanism.

Elastic relaxation in ice has also been attributed to molecular reorientations, here induced by strains in the crystal;[12] the Bjerrum fault mechanism has been invoked to explain the necessary rearrangements.[13] Again a single relaxation time is defined experimentally[13] and theoretically,[11] the theoretical elastic relaxation time $\tau_e$ being related to $\tau_d$ by

$$\tau_d/\tau_e = 3/2,$$

in agreement with experiment. However, this ratio would be valid, whether or not the Bjerrum faults were paired with vacancies or interstitials, so this question remains unanswered.

Proton spin resonance experiments supply additional information about the motion of the protons. The temperature dependence of the spin-lattice relaxation time $T_1$ in pure ice[14, 15] is characteristic of relaxation by thermal motion of the spins and implies the same activation energy as that associated with the diffusion process (and dielectric and elastic relaxation).

Several authors[14–16] have correlated the spin-lattice relaxation with dielectric or elastic relaxation in ice; the relaxation mechanism assumed, then, has been the rotations produced by the Bjerrum fault migrations. Application of the theory of spin relaxation developed by Redfield,[17] however, shows that this mechanism would require $T_1$ (at $-2°$C and $\omega = 2\pi \times 30$ Mc/sec) to be at least 25 sec, whereas

the observed value is 7.5 sec. This suggests the existence of some faster motion not detected by the electrical or mechanical measurements;[18] since the diffusion of neutral species is indeed faster, this motion must be the primary one controlling the thermal equilibration of the proton spins.

In the case of ice, the predominant effect of diffusion on spin relaxation is the molecular rotation accompanying the translation; that is, the "intramolecular" contribution to $T_1^{-1}$ is large. This contribution can be calculated accurately for the various conceivable mechanisms for diffusion; after adding estimates of the effect of the more distant spins, the calculated relaxation times can be compared with the experimental value to judge the possible mechanisms.

The comparisons are decisive. Most of the conventionally invoked diffusion mechanisms—"direct" interstitial displacements, vacancy diffusion, or concerted jumps of neighbors—would entail a spin-lattice relaxation time of about a second, significantly shorter than observed. One diffusion mechanism, however, leads to a predicted $T_1$ consistent with experiment. This mechanism is the migration of "free" interstitials—molecules which diffuse several steps in the interstitial space before occupying a normal lattice site, in contrast with a "direct" interstitial displacement. From the observed relaxation time we can estimate the mean square displacement per trip to be about $8d^2$. This "free" interstitial mechanism for diffusion seems reasonable in view of the open structure.

In pure ice the spin-lattice relaxation effects of the electrically active motions are not completely overwhelmed by the neutral diffusion; the relative importance of the mechanisms may well be changed in doped samples, where soluble impurities alter the concentrations of the charged species. This appears to be confirmed in a general way by recent studies[19] of the proton spin-lattice relaxation in ice doped with HF and $NH_4F$.

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# *GENETIC EVIDENCE FOR THE SUBUNIT STRUCTURE OF LACTATE DEHYDROGENASE ISOZYMES**

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*Communicated by B. H. Willier, June 17, 1963*

The lactate dehydrogenases commonly occur in from one to five molecular forms (isozymes) in the various tissues of an animal species.[1] By means of zone electrophoresis, characteristic patterns of these isozymes may be demonstrated in each tissue. In adult animals, most tissues contain all five isozymes but in different proportions. Usually, heart muscle exhibits principally the first, or most negatively-charged, isozyme (LDH-1), while skeletal muscle and liver contain chiefly the most positively-charged (LDH-5).

Appella and Markert[2] treated crystalline lactate dehydrogenase from beef heart with 5 *M* guanidine-HCl. This reagent dissociates the LDH molecule into four subunits of equal molecular weight, as determined by sedimentation studies. These subunits, or polypeptide chains, were shown to exist in two different electrophoretic varieties which Markert[3] has designated as subunits A and B. Moreover, he theorized that the five isozymes are the five different tetramers that would be obtained by associating these two subunits in all possible combinations of four.[2, 3]

Cahn *et al.*,[4] using chicken LDH, showed that the two "pure" tetramers (LDH-1 and -5) are immunologically distinct and, further, that the three intermediate isozymes (LDH-2, -3, and -4) are cross-reactive with both pure types. Thus, these intermediate isozymes presumably contain both kinds of subunits. Recently, Markert[5] dissociated LDH-1 and LDH-5 into subunits by freezing in 1 *M* NaCl; after thawing, the subunits recombined at random in groups of four to yield all five isozymes in approximately the expected ratio of 1:4:6:4:1. The postulated subunit formulae for the LDH isozymes makes these relationships clear: LDH-1 = BBBB, LDH-2 = BBBA, LDH-3 = BBAA, LDH-4 = BAAA, LDH-5 = AAAA.

It has been tacitly assumed that the two different polypeptides, A and B, are under separate genetic control. This study presents genetic data which support this assumption. It also reinforces the concept of a tetrameric structure of the LDH molecule.

*Materials and Methods.*—The animals were obtained from a laboratory population of deer mice, *Peromyscus maniculatus*. All animals studied were at least 2 months old, and females were nonpregnant and nonlactating at the time of study.

The mice were killed by decapitation, and tissues to be analyzed were removed immediately, washed in cold 0.9% saline solution, blotted, weighed, and homogenized in a glass homogenizer with the appropriate amount of cold distilled water, which was usually 3–10 times the volume of the tissue. Liver, kidney, brain, and testis were routinely studied. The homogenate was placed in a polypropylene centrifuge tube, frozen and thawed twice, and centrifuged at 25,000 × *g* for ten min at 4–8°C. The clear supernatant was removed, and 40 microliters were added to the starch-gel insert. Vertical electrophoresis was carried out for 18 hr at a voltage

gradient of 10 volts per centimeter at 4°C. A continuous borate buffer system was used, and the gel buffer was 0.015 *M* at pH 8.4.

The gel was sliced horizontally into halves and incubated for 2 hr at 35°C in 100 ml of a solution containing 40% sodium DL-lactate 10 ml, DPN 25 mg, phenazine methosulfate 2 mg, nitro blue tetrazolium 25 mg, and 0.5 *M* tris, pH 7.1, 10 ml. Zones of LDH activity appeared as purple bands.

A screening study was carried out on individuals from a number of *Peromyscus* families kept in the laboratory, and a variant pattern of LDH, subsequently shown to be that of a heterozygote, was found in two related sibships. One of these belonged to the subspecies *P. maniculatus bairdi*, the other came from a cross between *P. m. bairdi* and a subspecific hybrid. Brother-sister matings of the offspring and backcrosses with both parents were carried out. Since it was necessary to sacrifice the animals before testing them for enzyme activity, the crosses had to be made "blind," hoping that, by chance, some of the mated animals would contain the variant pattern. Brain and kidney from each animal were analyzed, and animals of any one genotype invariably showed identical patterns except for minor quantitative differences.

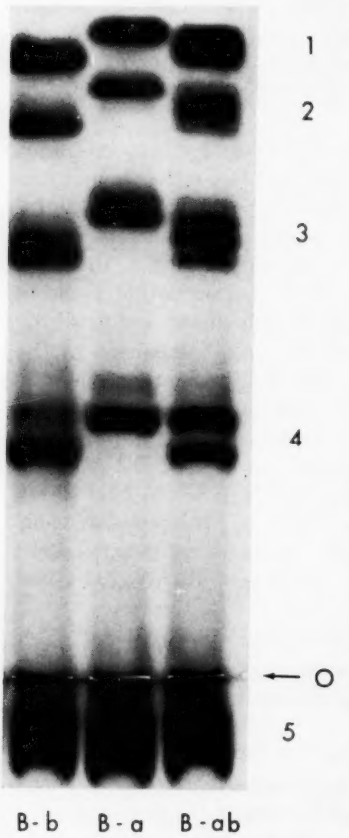


FIG. 1.—Zone electrophoresis patterns of *Peromyscus* brain extract on starch gel, showing three phenotypes of LDH isozymes: B-a normal, B-b homozygous variant, and B-ab heterozygous. Anode is at top, origin is at 0. Several extra or "ghost" bands are seen just anodal to some major bands, most prominent in this gel at Band 3. These occur inconstantly and are believed to represent *in vitro* artifacts.

*Results.*—Three patterns of LDH isozymes were observed (Fig. 1) involving variations in Bands 1, 2, 3, and 4, but not 5. In the normal pattern, here called B-a, and in the one considered to be the homozygous variant, B-b, the bands are similar in intensity and configuration, but the first four bands of this variant migrate more slowly than the corresponding bands in the normal mice. In the heterozygote, B-ab, Band 4 has 2 components of equal intensity, Band 3 is a triplet with the middle of the three being darker than the other two, Band 2 occurs as four components, the two middle ones being darkest, and Band 1 appears as a single, wide, diffuse zone which should theoretically contain five components. At each isozymic position the fastest component in the heterozygote lies parallel to the corresponding band in normal mice, while the slowest component advances at the same rate as the corresponding band of the homozygous variant.

Offspring have been obtained from three of the six possible mating types: B-a x B-a, B-a x B-ab, and B-ab x B-ab. The results of the matings are shown in Table 1. They demon-

TABLE 1

RESULTS OF GENETIC ANALYSIS

| Mating type | Number of sibships | Number of offspring | Phenotypes of Offspring: Normal Male | Normal Female | Heterozygote Male | Heterozygote Female | Homozygous variant Male | Homozygous variant Female | Ratios of Phenotypes (normal:heterozygote:homozygous variant) Expected | Observed |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal × normal | 2 | 24 | 13 | 11 | 0 | 0 | 0 | 0 | 24:0:0 | 24:0:0 |
| Normal × heterozygote | 6 | 54 | 12 | 15 | 11 | 16 | 0 | 0 | 27:27:0 | 27:27:0 |
| Heterozygote × heterozygote | 2 | 12 | 2 | 1 | 0 | 5 | 2 | 2 | 3:6:3 | 3:5:4 |

strate that the variations in LDH pattern segregate in the expected Mendelian ratios. The six crosses between normals and heterozygotes included five matings involving heterozygous males. Among their progeny, which totaled 51, males and females were distributed approximately equally among the two expected classes. This demonstrates that the inheritance is autosomal.

*Terminology.*—The symbol *L-1* is suggested for the locus involved; *L* indicates lactate dehydrogenase, and the number one denotes the first locus involved in this group of enzymes discovered in the deer mouse. The normal gene at this locus is designated $L\text{-}1^a$, and its product is the $B^a$ polypeptide. The variant allele is $L\text{-}1^b$, producing polypeptide $B^b$. This system retains the B notation for the polypeptide, as established by Markert.[3] Any subsequently discovered alleles would be designated by higher superscript letters. The genotypes, their polypeptide products, and phenotypes of the three LDH patterns would be as follows:

| | *Genotype* | *Polypeptides produced* | *Phenotype* |
|---|---|---|---|
| Normal | $L\text{-}1^a/L\text{-}1^a$ | $B^a$ | B-a |
| Heterozygote | $L\text{-}1^a/L\text{-}1^b$ | $B^a$, $B^b$ | B-ab |
| Homozygous variant | $L\text{-}1^b/L\text{-}1^b$ | $B^b$ | B-b |

*Discussion.*—The findings indicate that LDH Bands 1–4 in *Peromyscus* share a common polypeptide which is under the control of an autosomal locus, and that Band 5 does not contain this polypeptide. The variant thus involves an alteration of the B polypeptide.

In the heterozygote, the two allelic genes presumably produce equal amounts of their respective polypeptides, and the tetrameric molecules would be composed in part of "hybrid" forms produced by combinations of the normal and altered polypeptides, $B^a$ and $B^b$. The theoretical structures of the various isozymes in the three types of patterns, with the expected binomial proportions of each, assuming random combination, are shown in Table 2.

It can be seen in Figure 1 that Bands 2–5 fit the above scheme. Band 1 appears in the heterozygote as a single diffuse zone, and is presumed to contain five bands which were not resolvable in the electrophoretic system used in this investigation. The findings thus support the hypothesis of a tetrameric structure of the LDH isozymes, the molecules being composed of various combinations of two distinct polypeptides (A and B) which are under separate genetic control.

Whether the variant LDH molecules confer any selective advantage or disadvantage is not evident. The enzyme activity of the altered forms is apparently unchanged, and the homozygous animals, both normal and variant, appeared healthy.

TABLE 2

PROPOSED TETRAMERIC STRUCTURES OF THE THREE PATTERNS OF LDH ISOZYMES SHOWING RELATIVE POSITIONS OF SUB-BANDSWITHIN EACH MAJOR BAND

| | Normal (genotype *L-1a/L-1a*) | Heterozygote* (genotype *L-1a/L-1b*) | Homozygous Variant (genotype *L-1b/L-1b*) |
|---|---|---|---|
| LDH-1 | $B^a$ $B^a$ $B^a$ $B^a$ | $^1/_{16}$ $B^a$ $B^a$ $B^a$ $B^a$ | |
| | | $^4/_{16}$ $B^a$ $B^a$ $B^a$ $B^b$ | |
| | | $^6/_{16}$ $B^a$ $B^a$ $B^b$ $B^b$ | |
| | | $^4/_{16}$ $B^a$ $B^b$ $B^b$ $B^b$ | |
| | | $^1/_{16}$ $B^b$ $B^b$ $B^b$ $B^b$ | $B^b$ $B^b$ $B^b$ $B^b$ |
| LDH-2 | A $B^a$ $B^a$ $B^a$ | $^1/_8$ A $B^a$ $B^a$ $B^a$ | |
| | | $^3/_8$ A $B^a$ $B^a$ $B^b$ | |
| | | $^3/_8$ A $B^a$ $B^b$ $B^b$ | |
| | | $^1/_8$ A $B^b$ $B^b$ $B^b$ | A $B^b$ $B^b$ $B^b$ |
| LDH-3 | A A $B^a$ $B^a$ | $^1/_4$ A A $B^a$ $B^a$ | |
| | | $^2/_4$ A A $B^a$ $B^b$ | |
| | | $^1/_4$ A A $B^b$ $B^b$ | A A $B^b$ $B^b$ |
| LDH-4 | A A A $B^a$ | $^1/_2$ A A A $B^a$ | |
| | | $^1/_2$ A A A $B^b$ | A A A $B^b$ |
| LDH-5 | A A A A | A A A A | A A A A |

* Subunit polypeptides are A, $B^a$, and $B^b$. Ratios preceding the heterozygous tetramers indicate relative proportions of each sub-band within that major band.

*Summary.*—A genetically-determined variant pattern of LDH isozymes was found in kidney and brain extracts of certain stocks of the deer mouse, *Peromyscus maniculatus*. Analysis by starch-gel electrophoresis shows that four of the five isozymic bands are involved, the altered molecules migrating more slowly than the normal ones. Heterozygous individuals contain the normal and variant bands, as well as additional ones formed by the combination of normal and altered polypeptides in the same molecule.

The frequency distribution of the various LDH patterns among offspring from different types of matings demonstrates that the observed variations are genetically determined, involving a single autosomal locus which controls the polypeptide found in the four most negatively-charged isozymes. In addition, the findings conform with the hypothesis that the LDH isozymes are tetramers composed of the five possible combinations of two different subunits.

*Note added in proof:* LDH-1 in the heterozygote has now been resolved into five bands, in approximately the expected proportions of 1:4:6:4:1. This was done by increasing the gel buffer strength to 0.1 *M*. Also, by using a phosphate buffer system (0.01 *M*, pH 8.5), LDH-5 has been caused to migrate anodally. It occurs as a single, discrete band, and clearly shows no genetic variation in this study.

The authors wish to acknowledge the helpful suggestions of Prof. Clement L. Markert of the Johns Hopkins University, and the excellent technical assistance of Mrs. Lucille Setter.

* This investigation was supported in part by a grant from the Scottish Rite Foundation for Research in Schizophrenia, and by USPHS research grant NB-03095, National Institutes of Health.

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# *COLLAGEN IN FOSSIL BONE**

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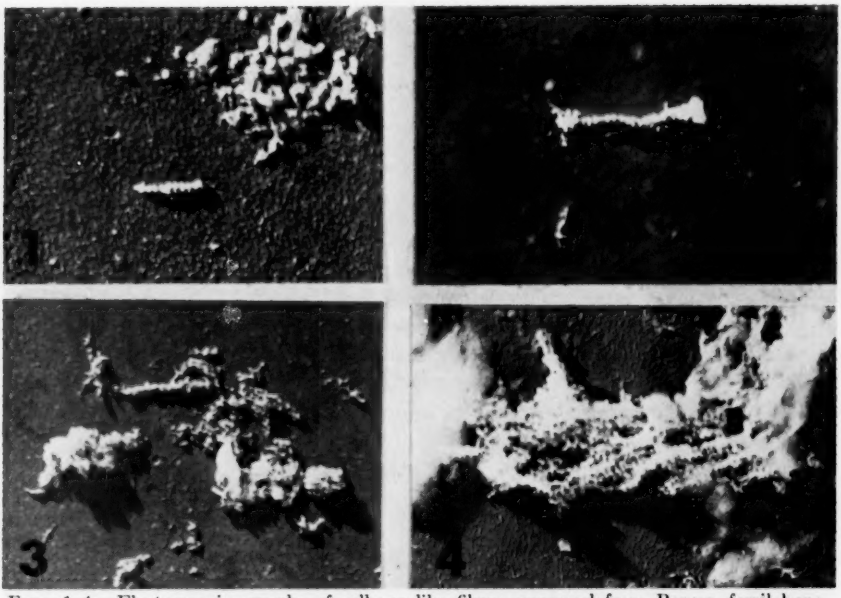
*Communicated June 24, 1963*

Amino acids have frequently been demonstrated[1] in acid solutions of well-preserved fossils, some of great age. They are undoubtedly often fragments of proteins that originally were present but whose inherent instability is such that they do not persist over geologically significant periods of time. Nevertheless, there are reports of collagen found in mummified human remains[2] several thousand years old, and in mastodon bones[3] preserved under permafrost conditions; and recently it has been stated[4] that this protein has been identified in the fossilized antlers of a deer-like animal of Miocene age (±30,000,000 years). Evidently it is of fundamental importance to determine if any protein, considered as the basic material of all living organisms, can endure, for its detailed examination and analysis could make substantial contributions to our knowledge of evolutionary processes.

The search for such ancient proteins is one aspect of the broader study that can now be made of the microstructure and composition of the fossilized remains of earlier forms of life. Microradiography using X-rays of appropriate wavelengths can establish at the microscopic level whether original structures have been preserved or have been replaced through mineralization; X-ray spectroscopy and diffraction can determine the nature of whatever replacement has occurred. In the case of fossilized teeth, bones, and shells, the calcium phosphate and carbonate that were their original minerals can be selectively removed by chemical means, and microanalyses made of both the organic and inorganic components of the residues thus obtained.

The present paper is a preliminary account of the nature of carbonaceous residues from Early Pleistocene bones collected from the Mendivil Ranch locality (Benson fauna) in Southern Arizona. The specimen analyzed in detail was the heel bone (calcaneum) from an animal of the genus Equus[5].

All samples chosen for study were ones whose fragments were positive for carbon when charred in the absence of oxygen, and whose residues showed no optical or electron microscopic evidence of recent microorganismal invasion. This examination for invasion has been found essential because experience shows that in a fossil the presence of actively growing molds may not be macroscopically apparent. Obviously, subsequent analysis for protein or amino acids is meaningless if such organisms are present. Thick sections of cleaned bone were decalcified by prolonged treatment at room temperature in 1–2.5% trichloroacetic acid, sometimes in the presence of 4% formaldehyde to prevent attack by airborne bacteria. For electron microscopy the residues from this acid treatment, with pulverization either before or after decalcification, were washed repeatedly by centrifugation. A final suspension in water was dried down on the usual collodion-covered grids, shadowed with chromium, and strengthened for microscopy by the vertical evaporation of a thin layer of carbon. Examination was carried out with a small Philips EM75 electron microscope. Fields rich in fibrous material were common, and many of these




FIGS. 1–4.—Electron micrographs of collagen-like fibers recovered from Benson fossil bone. Magnification 16,500 ×.

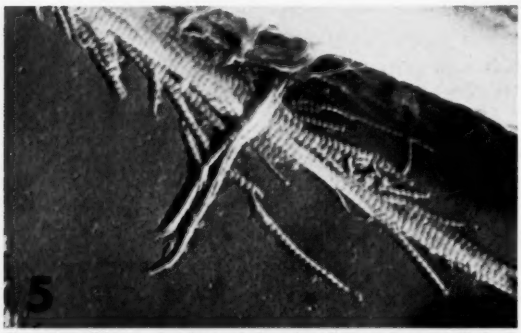



FIG. 5.—Electron micrographs of collagen recovered from desert-dried recent bone. Magnification 16,500 ×.

fibers have a type of regularly striated fine structure (Figs. 1–4) strongly suggestive of collagen (Fig. 5).

Portions of the residues shown by preliminary analysis to contain about two mg of nitrogen were hydrolyzed for chromatography by heating at *ca.* 100°C for 24 hr in 6 *N* HCl followed by evaporation to dryness over hot water. A solution in $H_2O$ of this residue was poured onto a Dowex 50W-X2 column, and the nonadsorbed cations removed by repeated washings with water. Adsorbed amino acids and peptides, if present, were then eluted by the cautious addition of dilute $NH_4OH$. The eluate was dried and freed of ammonia by evaporation over hot water; its amino acid content was then determined by unidimensional paper chromatography using phenol as partitioning and ninhydrin as coloring agents. Several controls were run. One type consisted of fossil bones[6] from the tar pits of La Brea; they were chosen because they are of Pleistocene age, though much younger than the Benson bones, and because the condition of their preservation should be better. After extraction with petroleum ether and the removal of impregnating sand, samples

of the La Brea bone were decalcified in dilute HCl and the residues hydrolyzed as outlined above. Other controls were fresh rat tail tendon, a fresh human tooth, a recent sea lion tooth, and desert-dried calf bone. All these controls, including the La Brea residue, yielded excellent chromatograms which were qualitatively alike and showed the presence of at least the following amino acids: glycine, hydroxyproline, proline, alanine, leucine, glutamic acid, and aspartic acid.

Benson hydrolysates prepared from several samples invariably resulted in definite but weak chromatograms suggestive of about four of the above amino acids. Since their spots cannot be due to free amino acids which would have been eliminated in the original acid solution of the bone, two possibilities exist. They could be derived from an incomplete, insoluble (polypeptide) residue of the initial collagen, or there could be so little collagen remaining in the fossil that a larger sample was needed to provide a complete chromatogram. This second alternative proved correct for, starting with eight gm of fossil bone and employing a more concentrated eluate, a chromatogram was obtained which was qualitatively like those yielded by the controls. It would appear that considerably less than one per cent of the initial collagen remained in the Benson bones. This amount of collagen seemed too small to account satisfactorily for the numerous striated fibers present in the electron microscopic preparations. As Figures 1–4 indicate, the fossil fibers resemble collagen in general appearance and periodicity, but there are significant differences. To facilitate comparison, electron micrographs were made of decalcified fragments of the desert-dried bone. The collagen they contain (Fig. 5) appears identical with fresh collagen. The fossil fibers of Figures 1–3 are markedly shrunken, except longitudinally, compared to those of Figure 5; Figure 4 is typical of large masses of fibers in which the bands are still more reduced in thickness. The repetition of banding along the fossil fibers is *ca.* 600 A compared with the normal 640 A collagen spacing. In the electron micrographs of our fossil material, all sorts of transitions between these shrunken fibers are visible, but Figure 1 was chosen to show as close an approach to fresh collagen as has been observed in the Benson fossils. It is hard to avoid the conclusion that the fossil fibers are residual collagen altered with the passage of time.

This raises the important question of whether the change is merely a shrinkage due to a gradual disappearance of much of the initial protein or involves a substitution of inorganic matter. In a preliminary attempt to decide between these alternatives, the residues were examined by X-ray spectroscopy and diffraction. Powder photographs[7] showed only some clay and a pattern probably attributable to an oxide of uranium, which the spectroscopy demonstrated to have been highly concentrated from the trace detectable in the original bone. The X-ray spectroscopy revealed, besides this uranium, only some iron and calcium possibly associated with the clay. Additional work will be required to show their relation, if any, to the remaining organic matter.

When fresh bone is demineralized by the kind of treatment employed here, there remains a jelly which, though rich in collagen, cannot easily be shredded to free its individual fibers for electron microscopic identification; these are more readily seen in embedded and thinly sectioned preparations. Unlike the Benson material, the La Brea residues behave in similar fashion and, though collagen is demonstrable under the electron microscope, sectioned preparations are needed with them also.

Thus, the present preliminary observations demonstrate that the La Brea collagen is less altered than that in the Benson fossils, but they do not indicate if this should be attributed to their younger age or to the better conditions of preservation that undoubtedly prevailed.

These studies, as extended to bones and teeth of various ages, will be described elsewhere in greater detail.

* This work has been supported by grants from the National Science Foundation (grant 9760) and the National Institutes of Health (grant DE-01183).

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[5] We are indebted to Drs. John F. Lance and Paul A. Wood of the Geology Department, University of Arizona, for these fossils and their identification.

[6] We wish to thank Drs. Theodore Downs and Reid McDonald of the Los Angeles County Museum for the La Brea fossils.

[7] The diffraction photographs were prepared by Victor J. Hoffmann.

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## *ON THE STRUCTURE OF COMPACT COMPLEX ANALYTIC SURFACES*

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*Communicated by D. C. Spencer, June 10, 1963*

The purpose of this note is to outline our recent results on the structure of compact complex analytic surfaces. Details will be published elsewhere. Our proof of the results is based on the Riemann-Roch theorem of which the complete form has been established recently by M. F. Atiyah and I. M. Singer.[1]

1. We denote by $\mathbf{Z}$ the ring of rational integers and by $\mathbf{C}$ the field of complex numbers. By a *surface* we shall mean a compact complex analytic surface free from singularities. We consider a surface $S$ and denote by $b_\nu$ the $\nu$-th Betti number of $S$ and by $c_\nu$ the $\nu$-th Chern class of $S$. In view of the isomorphism $H^4(S, \mathbf{Z}) \cong \mathbf{Z}$, we may consider ${c_1}^2$ and $c_2$ as rational integers. Letting $\{\Gamma_1, \ldots, \Gamma_i, \ldots, \Gamma_{b_2}\}$ denote a Betti base of 2-cycles on $S$, we define $b^+$ and $b^-$ to be, respectively, the number of positive and negative eigenvalues of the symmetric matrix $(\Gamma_i\Gamma_k)$, where the symbol $(\Gamma_i\Gamma_k)$ denotes the intersection multiplicity of $\Gamma_i$ and $\Gamma_k$. Then we have[2]

$$b^+ - b^- = -\frac{2}{3}c_2 + \frac{1}{3}{c_1}^2, \qquad b^+ + b^- = b_2. \tag{1}$$

We denote by $\mathcal{O}$ and by $\Omega^r$, respectively, the sheaves over $S$ of germs of holomorphic functions and of holomorphic $r$-forms. We define

$$h^{r,\nu} = \dim H^\nu(S,\Omega^r), \qquad \Omega^0 = \mathcal{O}.$$

We set $p_g = h^{0,2} = h^{2,0}$, $q = h^{0,1}$, and call $p_g$ and $q$, respectively, the *geometric genus* and the *irregularity* of $S$. We denote by $K$ the *canonical bundle* on $S$. Note that the Chern class of $K$ is equal to $-c_1$. The surface $S$ is said to be *regular* if and only if the irregularity $q$ vanishes. We have the exact sequence

$$0 \to \mathbf{C} \to \mathcal{O} \xrightarrow{d} d\mathcal{O} \to 0$$

and the corresponding exact cohomology sequence

$$0 \to H^0(S, d\mathcal{O}) \to H^1(S, \mathbf{C}) \to H^1(S, \mathcal{O}) \to \dots, \tag{2}$$

where $d\mathcal{O}$ is the sheaf over $S$ of germs of $d$-closed holomorphic 1-forms.

LEMMA 1. *Every holomorphic* 1*-form defined on* $S$ *is* $d$*-closed.*

LEMMA 2. *If* $d$*-closed holomorphic* 1*-forms* $\varphi_1, \varphi_2, \dots, \varphi_h$ *defined on* $S$ *are linearly independent, then the* $d$*-closed* 1*-forms* $\varphi_1, \varphi_2, \dots, \varphi_h, \bar{\varphi}_1, \dots, \bar{\varphi}_h$ *are* $d$*-cohomologically independent.*

We infer from Lemmas 1 and 2 that

$$h^{1,0} = dim\ H^0(S, d\mathcal{O}) \leqq {}^1/_2\, b_1. \tag{3}$$

Hence, we obtain from (2) the inequalities

$$q \geqq b_1 - h^{1,0} \geqq {}^1/_2\, b_1. \tag{4}$$

Moreover, we have

$$b^+ \geqq 2p_g. \tag{5}$$

The Riemann-Roch theorem implies the equality

$$12(p_g - q + 1) = c_1^2 + c_2. \tag{6}$$

Combining this with (1) we get

$$2q - b_1 + b^+ - 2p_g = 1,$$

while (4) and (5) show that $2q - b_1$ and $b^+ - 2p_g$ are both nonnegative integers. Consequently, we obtain

THEOREM 1. *If* $b_1$ *is even, then* $2q = b_1$, $b^+ = 2p_g + 1$, *and* $h^{1,0} = q$. *If* $b_1$ *is odd, then* $2q = b_1 + 1$, $b^+ = 2p_g$ *and* $h^{1,0} = q - 1$.

2. By an algebraic surface we mean a nonsingular algebraic surface imbedded in a projective space (of any dimension). By an *elliptic surface* we mean an analytic fiber space of elliptic curves over an algebraic curve. *If there exist on* $S$ *two algebraically independent meromorphic functions, then* $S$ *is an algebraic surface. If there exists on* $S$ *one and only one algebraically independent meromorphic function, then* $S$ *is an elliptic surface.*[3]

For any complex line bundle $F$ over $S$, we denote by $\mathcal{O}(F)$ the sheaf of germs of holomorphic sections of $F$, and by $c(F)$ the Chern class of $F$. The Riemann-Roch theorem implies the inequality

$$dim\ H^0(S, \mathcal{O}(mF)) + dim\ H^0(S, \mathcal{O}(K - mF)) \geqq \frac{1}{2}\, m^2c^2 + \frac{1}{2}\, mc_1c + \frac{1}{12}\,(c_1^2 + c_2), \tag{7}$$

where $m$ is an arbitrary integer and $c = c(F)$. We infer from this inequality that, *if there exists on S a complex line bundle F with* $c(F)^2 > 0$, *then* S *is an algebraic surface.* In particular we have

THEOREM 2. *If* $c_1^2 > 0$, *then S is an algebraic surface.*

If $p_g \geqq 2$, then there exist on $S$ nonconstant meromorphic functions. Hence, we get

THEOREM 3. *If* $p_g \geqq 2$, *then S is either an algebraic surface or an elliptic surface.*

Moreover, we have

THEOREM 4. *If* $h^{1,0} \geqq 3$, *then S is either an algebraic surface or an elliptic surface.*[4]

Now we consider the case in which $b_1$ is even and $p_g = 0$. Since, by Theorem 1, $b^+ = 1$, there exists a cohomology class $c \in H^2(S, \mathbf{Z})$ with $c^2 > 0$, and the vanishing of $p_g$ implies the existence of a complex line bundle $F$ over $S$ with $c(F) = c$. Consequently, we obtain

THEOREM 5. *If* $b_1$ *is even and if* $p_g = 0$, *then S is an algebraic surface.*

3. Any surface is obtained from a surface containing no exceptional curve (of the first kind) by means of several quadric transformations.[5] Note that the first Betti number, the irregularity, and the geometric genus of a surface are invariant under any quadric transformation. In this section we assume that the surface $S$ has no meromorphic function (except constants) and contains no exceptional curve.

(*A*) The case in which $b_1$ is even. It follows from Theorems 1, 3, 4, and 5 that $b_1 = 2q$, $q = h^{1,0} \leqq 2$, and $p_g = 1$. Hence, we conclude in the same manner as in the case of Kähler surfaces[6] that $q$ is equal to either 2 or 0, that, if $q = 2$, the surface $S$ is a complex torus, and that, if $q = 0$, the canonical bundle $K$ on $S$ is trivial. Following A. Weil, we call a regular surface with trivial canonical bundle a $K_3$ *surface.* In case $q = 0$, the surface $S$ is, then, a $K_3$ surface. We note that the second Betti number of any $K_3$ surface is equal to 22.

(*B*) The case in which $b_1$ is odd. It follows from Theorems 1, 2, 3, and 4 that $b_1 = 2q - 1$, $q = h^{1,0} + 1$, $b^+ = 2p_g$, $h^{1,0} \leqq 2$, $p_g \leqq 1$, and $c_1^2 \leqq 0$. We have

LEMMA 3. *If* $p_g = 1$, *then* $c_1^2 = 0$.

(*i*) If $h^{1,0} = 2$, then $q = 3$, $b_1 = 5$, and there exist two linearly independent holomorphic 1-forms $\varphi_1, \varphi_2 \in H^0(S, d\mathfrak{O})$. Moreover, the exterior product $\varphi_1 \wedge \varphi_2$ does not vanish identically. Hence, $p_g = 1$ and therefore, by Lemma 3, $c_1^2 = 0$. The formula (6) then proves that $b_2 = -4$. This is a contradiction.

(*ii*) If $h^{1,0} = 1$, then $q = 2$ and $b_1 = 3$. We take a holomorphic 1-form $\varphi \in H^0(S, d\mathfrak{O})$, $\varphi \neq 0$, and find a 1-form $\sigma$ of type (1, 0) defined on $S$ such that $d\sigma = \varphi \wedge \bar{\varphi}$ and such that $\sigma + \bar{\sigma}$, $\varphi$ and $\bar{\varphi}$ generate the $d$-cohomology group of 1-forms on $S$. We then obtain multivalued holomorphic functions $w$ and $z$ on $S$ satisfying the differential equations

$$dw = \sigma + \bar{z}\varphi, \qquad dz = \varphi.$$

We infer that the exterior product $dw \wedge dz$ never vanishes. Hence, the space $\mathbf{C}^2$ of the complex variables $w$ and $z$ forms the universal covering surface of $S$. The covering transformation group of $\mathbf{C}^2$ over $S$ is generated by the linear transformations

$$g_\nu : w \to w + \bar{\alpha}_\nu z + \beta_\nu,\ z \to z + \alpha_\nu,\ \nu = 1, 2, 3, 4$$

such that

$$\alpha_3 = \alpha_4 = 0, \qquad \Re\beta_3 \neq 0, \qquad \bar{\alpha}_1\alpha_2 - \bar{\alpha}_2\alpha_1 = m\,\beta_4,$$

where $m$ is a positive integer. It follows that $S$ is an elliptic surface. This is contrary to the assumption that $S$ has no meromorphic function (except constants).

*(iii)* If $h^{1,0} = 0$, then $q = 1$, and $b_1 = 1$. Therefore, the Picard variety $\mathcal{P} = H^1(S, \mathcal{O})/H^1(S, \mathbf{Z})$ is isomorphic to the Lie group $\mathbf{C}/\mathbf{Z}$. We shall show that $p_g = 0$. Suppose that $p_g = 1$. Then, for any complex line bundle $P \in \mathcal{P}$, the inequality

$$dim\ H^0(S, \mathcal{O}(P)) + dim\ H^0(S, \mathcal{O}(K - P)) \geqq 1$$

holds. It follows that there exist infinitely many irreducible curves on $S$. This contradicts the assumption that $S$ has no meromorphic function[7] (except constants).

Thus, we obtain

THEOREM 6. *Assume that $S$ has no meromorphic function except constants and contains no exceptional curve. If $b_1$ is even, then $S$ is either a complex torus or a $K_3$ surface. If $b_1$ is odd, then $b_1 = q = 1$, and $p_g = 0$.*

4. We infer from the above results that, *if $b_1$ is even, then $S$ is an algebraic surface or an elliptic surface, or $S$ is obtained from a complex torus or a $K_3$ surface by means of several quadric transformations. If $b_1$ is odd, then either $S$ is an elliptic surface, or $b_1 = q = 1$, and $p_g = 0$.* Complex tori and $K_3$ surfaces are deformations of algebraic surfaces,[8] while an elliptic surface is a deformation of an algebraic surface if and only if its first Betti number is even.[9] Hence, we conclude:

THEOREM 7. *A compact complex analytic surface $S$ is a deformation of an algebraic surface if and only if the first Betti number $b_1$ of $S$ is even.*

THEOREM 8. *If the first Betti number $b_1$ of $S$ is odd, then $S$ is an elliptic surface, except the case in which $b_1 = q = 1$, and $p_g = 0$.*

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[3] Kodaira, K., "On compact complex analytic surfaces, I," *Annals of Math.*, **71**, 111–152 (1960).

[4] *Ibid.*, section 5.

[5] Grauert, H., "Über Modifikationen und exzeptionelle analytische Mengen," *Math. Ann.*, **146**, 331–368 (1962).

[6] Kodaira, K., *op. cit.*, section 5.

[7] Kodaira, K., *op. cit.*, Theorem 5.1.

[8] The structure of $K_3$ surfaces has been studied by A. Weil, A. Andreotti, and H. Grauert.

[9] Kodaira, K., "On compact analytic surfaces, III," to appear in the *Annals of Math.*

# *INVARIANT IMBEDDING AND RAREFIED GAS DYNAMICS**

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*Communicated by S. Chandrasekhar, May 21, 1963*

1. *Introduction.*—The use of invariance principles and invariant imbedding techniques, originating in the work of Ambarzumian and Chandrasekhar, has provided new insights and computational solutions to various problems in the fields of radiative transfer and neutron transport theory.[1] Let us indicate how these same techniques can be applied to representative problems in the kinetic theory of gases. The possibility of this is suggested by the essential similarity of all particle processes: the behavior of neutrons or photons, or the structureless spherically symmetric molecule of the classical kinetic theory. For the present, we shall restrict our area of interest to linearized scattering models, finite collision frequencies, and relatively simple geometries. We leave to future study the application of invariance techniques to nonlinear scattering models and to cases in which the Boltzmann gain and loss operators are only conditionally convergent. A classic problem, that of plane shear flow between two infinite flat plates, will be used to present the ideas of invariant imbedding in the context of rarefied gas dynamics.

2. *Kinetic-Theory Approach.*—Various kinetic-theory approaches to the problem of plane shear flow are described in some detail by Willis.[2] The physical picture is the following.

An infinite plane wall moves with a small constant velocity $V_w$ in its plane at a distance $d$ from a fixed wall. Both walls are maintained at the same temperature, and may consist of either similar or dissimilar materials. It is desired to find the gas velocity and the stress at the moving wall as a function of the degree of rarefaction, which is measured by an appropriate Knudsen number. In the body of the gas, we shall use the single relaxation time model of Krook[3] in its linear version. Linearization is made possible by requiring that the Mach number of the moving wall be much less than unity, and then using standard perturbation techniques.

We define the following quantities:

$$n = \iint_{-\infty}^{+\infty} \int f d\vec{v}, \quad \tau = \delta n_0 \sqrt{h_0}\, d, \quad \bar{c} = \sqrt{\bar{u}^2 + \bar{v}^2 + \bar{w}^2},$$

$$n\bar{V} = \iint_{-\infty}^{+\infty} \int f \bar{v} d\vec{v}, \quad u, v, w = \sqrt{h}\,\bar{u}, \sqrt{h}\,\bar{v}, \sqrt{h}\,\bar{w},$$

$$3n/2h = \iint_{-\infty}^{+\infty} \int f(\bar{v} - \bar{V})^2 d\vec{v}, \quad y = x/d. \tag{2.1}$$

The nonlinear Krook[3] equation for this one-dimensional geometry can be written

$$\frac{\bar{u} df}{dx} = -\delta n \left[ f - n \left( \frac{h}{\pi} \right)^{3/2} \exp\left(-h(\bar{c} - \bar{V})^2\right) \right]. \tag{2.2}$$

The linearized version of this equation, obtained by setting $\sqrt{h}\,\bar{V}_w \ll 1$,[4] (where $V_w$ is the velocity of the moving wall) is

$$\frac{\bar{u} df}{dx} = -\delta n \left[ f - n \left( \frac{h}{\pi} \right)^{3/2} \exp\left(-h\bar{c}^2\right)(1 + 2h\bar{v}\,\bar{V}) \right]. \tag{2.3}$$

If $f = n_0 \left(\frac{h_0}{\pi}\right)^{3/2} \exp(-h_0\bar{c}^2) + f_1$, where $f_1 = n_0 \left(\frac{h_0}{\pi}\right)^{3/2} \exp(-h_0\bar{C}^2) v\Phi(u, x)$,

then the equation for the perturbation distribution function may be written

$$\frac{u df_1}{dy} = \tau \left[ - f_1 + \frac{2e^{-c^2}}{\pi^{3/2}} v \int_{-\infty}^{+\infty} \iint f_1 \, v \, du dv dw \right]. \tag{2.4}$$

The boundary conditions corresponding to purely diffuse reflection from the wall are

(*a*) Fixed plate: $f_1^+(x = 0) = 0$,

(*b*) Moving plate: $f_1^-(x=d) = n_0 \left(\frac{h_0}{\pi}\right)^{3/2} e^{-C^2} \cdot 2vM_w$, where $M_w = \sqrt{h}\, V_w$. (2.5)

It is possible to develop an equation for the function which is closely related to the radiative transport equation for isotropic scattering in a plane slab, i.e.,

$$\frac{u d\Phi}{dy} = \tau \left[ - \Phi + \frac{1}{\sqrt{\pi}} \int_{-\infty}^{+\infty} e^{-u^2} \Phi du \right]. \tag{2.6}$$

The diffuse boundary conditions associated with this equation are

$$y = 0, \Phi^+ = 0, y = 1, \Phi^- = 2\sqrt{h}\, V_w. \tag{2.7}$$

Willis has obtained a numerical solution to equation (6) by recasting it in a form similar to the Milne integral equation of radiative transfer. In addition, he has shown that straightforward Neumann iteration is suitable only for the very rarefied case, corresponding to $\tau < 1$. Since each subsequent calculation in the Neumann iteration scheme corresponds to higher-order collisions, it is not surprising that it is rather slowly convergent as the collision frequency is increased. This is quite similar to the state of affairs in radiative transfer, where it is known that a calculation based on successive absorption and scattering processes is ill-suited for computation as slab thickness is increased, or conversely, as the optical mean free path is decreased.

3. *Invariant-Imbedding Approach.*—The invariant-imbedding approach permits us to concentrate our attention on the values of quantities at the boundaries. This is particularly relevant to problems in heat transfer and aerodynamics, where values of certain molecular fluxes at the boundary are related to such macroscopic observables as energy transport and momentum transport to the walls. Once values have been obtained for the distribution function at the boundary, however, it is possible to calculate values of the distribution function within the body of the gas by relatively simple techniques.

The introduction of other boundary conditions besides the presently chosen diffuse re-emission is postponed so that we may focus on the essentials of the technique. In addition, it should be noted that our choice of one moving wall and one fixed wall is somewhat different from the usual Couette flow problem, where the two walls move in opposite directions. This choice was made because it obviated the need for the calculation of transmission functions. The method of linearization and the diffuse boundary conditions at the fixed wall result in the fixed wall acting as a

"sink" for perturbing molecules, in the sense that it emits only particles which possess the equilibrium distribution function.

Consider Figure 1. A reflection function $\rho(d, u, u_0', v, v_0', w, w_0')$ is defined which relates incoming molecules at $d$ to outgoing molecules at $d$:

$$f_1^+(u, v, w) = \int_0^{+\infty} \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} \rho(d, u, u_0', v, v_0', w, w_0') \cdot f_1^-(u_0', v_0', w_0')\, du_0' dv_0' dw_0'. \quad (3.1)$$

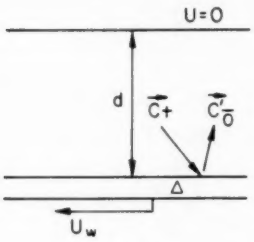


FIG. 1.

Linearity of the transport equation permits us to consider a Dirac $\delta$-function incident distribution centered about $u_0, v_0, w_0$. Thus, $\rho(d, u, u_0, v, v_0, w, w_0)$ is the outgoing distribution function of particles at $d$ due to an incident monochromatic beam at $d$ with velocity $u_0, v_0, w_0$. We observe that the sink-like character of the fixed diffuse wall guarantees that all outgoing perturbing particles at the moving wall are due solely to the disturbance generated by it. Simple particle-counting techniques are utilized to relate the reflection function $\rho(d, u, u_0, v, v_0, w, w_0)$ at $d$ to the reflection function $\rho(d + \Delta, u, u_0, v, v_0, w, w_0)$ at $d + \Delta$. We recognize that the original disturbing beam of molecules at $d + \Delta$ is modified by interactions within $\Delta$, and the resulting incoming distribution function at $d$ undergoes the same sort of processes at $d$ as the original beam underwent at $d + \Delta$. A new problem is initiated at $d$, which differs from the problem initiated at $d + \Delta$ by the fact that the incident distribution is continuous rather than monochromatic.

We introduce the mean free path as the unit of length, and use $\tau = \delta\, n_0 \sqrt{h_0}\, d$ rather than $d$ for the spacing between plates.

The spirit of the technique is exhibited in the simple particle-counting approach. To terms of order $(\Delta)^2$, $\rho(\tau + \Delta, u, u_0, v, v_0, w, w_0)$ is equal to the results of the following processes:[5]

(*a*) The incoming monochromatic beam is diminished by collisions in $\Delta$, reflected at $\tau$, and then diminished again by collisions in $\Delta$.

(*b*) The incoming monochromatic beam is scattered within $\Delta$ from $u_0, v_0, w_0$ to $u, v, w$.

(*c*) The incoming beam is reflected at $\tau$, and then scattered into $u, v, w$ within $\Delta$.

(*d*) The incoming beam is scattered within $\Delta$, then reflected at $\tau$ into $u, v, w$.

(*e*) The incoming beam is reflected at $\tau$, scattered in $\Delta$, and then reflected again at $\tau$.

Taking account of these interactions, we have

$$\rho(\tau + \Delta, u, u_0, v, v_0, w, w_0) = \left[1 - \frac{\Delta}{u}\right] \rho(\tau, u, u_0, v, v_0, w, w_0) \left[1 - \frac{\Delta}{u_0}\right]$$

$$+ \frac{2v}{(\pi)^{3/2} u} e^{-c_2^2} v_0 \Delta + 2e^{-c_2^2} \frac{v\Delta}{(\pi)^{3/2} u} \int_0^{\infty} \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} \rho(\tau, \dot{u}, u_0, \dot{v}, v_0, \dot{w}, w_0) \dot{v} d\dot{u} d\dot{v} d\dot{w}$$

$$+ \frac{2}{(\pi)^{3/2}} \Delta \int_0^{\infty} \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} e^{-c_2'^2} \frac{v_0 v'}{\dot{u}} \rho(\tau, u, \dot{u}, v, \dot{v}, w, \dot{w}) d\dot{u} d\dot{v} d\dot{w}$$

$$+ \frac{2}{(\pi)^{3/2}} \Delta \int_0^\infty \int_{-\infty}^\infty \int_{-\infty}^\infty \rho(\tau, u, \bar{u}, v, \bar{v}, w, \bar{w}) e^{-\bar{c}^2} \bar{v} d\bar{u} d\bar{v} d\bar{w}$$

$$\cdot \int_0^\infty \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} \rho(\tau, \dot{u}, u_0, \dot{v}, v_0, \dot{w}, w_0) \dot{v} \dot{u} d\dot{u} d\dot{v} d\dot{w}. \quad (3.2)$$

The requirements of section 2 suggest the transformation

$$\rho(\tau, u, u_0, v, v_0, w, w_0) = \frac{2}{\pi^{3/2}} e^{-\bar{c}^2} \frac{v v_0}{u} S(\tau, u, u_0). \quad (3.3)$$

Letting $\Delta \to 0$, we obtain the following symmetric form for the one-dimensional reflection function $S(d, u, u_0)$:

$$\frac{dS}{d\tau}(\tau, u, u_0) + \left(\frac{1}{u} + \frac{1}{u_0}\right) S(\tau, u, u_0) = 1$$

$$+ \frac{1}{\sqrt{\pi}} \int_0^\infty e^{-u^2_0} S(\tau, u, u_0) \frac{du_0}{u_0} + \frac{1}{\sqrt{\pi}} \int_0^\infty e^{-u^2_0} S(\tau, u, u_0) \frac{du}{u} \quad (3.4)$$

$$+ \frac{1}{\pi} \int_0^\infty e^{-u^2} S(\tau, \dot{u}, u_0) \frac{d\dot{u}}{\dot{u}} \int_0^\infty e^{-\bar{u}^2_0} S(\tau, u, \bar{u}_0) \frac{d\bar{u}_0}{\bar{u}_0}.$$

The initial condition associated with (4) is $S(O, u, u_0) = 0$, resulting from the observation that $\tau = 0$ corresponds to the free-molecule limit when there are no collisions within $d$ to reflect molecules from $u_0$ into $u$.

The last equation closely resembles the radiative-transport equation for isotropic scattering in a plane slab that Chandrasekhar[6] also obtains by invariance principles. A significant difference, however, between radiative transport and kinetic theory is that in kinetic theory, incoming distribution functions are not generally of a delta-function character, so that it is necessary to integrate over all incoming velocities in order to obtain the distribution function at the wall.

The computational solution of (4) has been readily obtained using techniques which were originally developed for problems in radiative transfer. In forthcoming publications we shall discuss this, consider the inclusion of nondiffuse particle-surface interaction at the moving wall, and present asymptotic observations which are relevant to the problems of near-free-molecule flow and slip flow.

* This research is supported by the Advanced Research Projects Agency under Contract No. SD 79. Any views or conclusions contained in this Memorandum should not be interpreted as representing the official opinion or policy of ARPA.

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[2] Willis, D. R., "Comparison of kinetic theory analyses of linearized Couette flow," *Phys. of Fluids*, **5**, 127–135 (1962).

[3] Bhatnagar, P. L., E. P. Gross, and M. Krook, "A model for collision processes in gases, I. Small amplitude processes in charged and neutral one-component systems," *Phys. Revi.*, **94**, 511–525 (1954).

[4] This is equivalent to requiring that the Mach number be everywhere small. $\sqrt{h}\, V_w$ is proportional to the Mach number of the wall.

[5] A consequence of the linearized Krook scattering model is that $\Delta\tau f_1(u, v, w)/u$ is the number of particles which are scattered out of the element of velocity space centered about $u$, $v$, $w$, and

$$\frac{\Delta\tau\ 2e^{-c^2v}}{(\pi)^{3/2}u}\int_{-\infty}^{+\infty}\int\int f_1 v_1 du_1 dv_1 dw_1$$

is the number of particles scattered into this element, all in an infinitesimal thickness $\Delta$.

[6] Chandrasekhar, S., *Radiative Transfer* (Oxford University Press, 1950).

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## *PREVENTION OF HORMONE ACTION BY LOCAL APPLICATION OF ACTINOMYCIN D*

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*Communicated by George W. Corner, June 12, 1963*

Previous work in this laboratory has suggested that growth hormone may exert its anabolic effect on liver by initial activation of RNA synthesis. Observations on the incorporation of labeled precursors revealed that after hormone administration the nuclear and microsomal fractions of RNA were increased significantly.[1] Short-pulse labeling experiments indicated that the stimulation was localized initially in the nuclear fractions of RNA.[2] On prolongation of the time of labeled precursor incorporation an increased specific activity appeared also in the microsome fraction. Nuclear RNA fractions from liver of normal and growth hormone-treated rats have been subfractionated into three components according to the method of Sibatani *et al.*[3] Sucrose gradient centrifugation studies on these fractions revealed maximum stimulation in a fraction associated with nucleolar RNA.[4] This fraction of RNA is a potent stimulator of amino acid incorporation into proteins in a cell-free system of *E. coli* of the type described by Matthaei and Nirenberg.[5] The growth hormone stimulation of RNA synthesis is inhibited by actinomycin D.[4] These results have prompted the suggestion that in exerting its hormonal effect, the primary action of growth hormone is the specific stimulation of messenger RNA synthesis and that this is a controlling prerequisite for secondary biologic effects such as cytoplasmic growth and cell multiplication. Evidence is now presented to suggest that the secondary cellular effects of two additional hormones, estradiol-17$\beta$ and chorionic gonadotrophin, depend on initial stimulation of messenger RNA synthesis. The work supports the hypothesis advanced by Hamilton in a recent communication[6] with respect to the action of estrogens.

*Materials and Methods.—Inhibition of estradiol action on vaginal mucosa:* Female rats of the AIIMS colony (derived from the Wistar strain) were ovariectomized at age 60–90 days, and the vaginal smears were followed daily to ascertain complete anestrus. After a priming dose of estradiol, the animals were allowed to revert to anestrus and were then used in the experiments. All animals were injected subcutaneously with 0.6 $\mu$g estradiol-17$\beta$ daily for four days, and the vaginal smears were recorded starting with the first day of injection. In the experimental groups, cotton plugs saturated with actinomycin D solution (0.5–5.0 $\mu$g) were inserted in the vagina and replaced twice daily. The control group underwent the same

procedure with 0.9% saline-saturated plugs. On the fourth day after initiating treatment the animals were sacrificed, the uteri were weighed on a torsion balance, and the vaginae fixed in Bouin's fixative for histologic preparation and study.

*Inhibition of gonadotrophin action on the testis:* Immature males ranging from 20 to 35 days of age[7] were utilized to test the effect of intratesticular actinomycin D on the response of the testis to systemically administered chorionic gonadotrophin. Each animal served as its own control. During the ten-day period of gonadotrophin administration (62.5 IU daily), the left testis was injected daily with 0.05 $\mu$g or 0.1 $\mu$g actinomycin D in a volume of 0.05 ml saline. The 27-gauge hypodermic needle was inserted through the scrotum and tunica albuginea, and the dosage delivered from a 0.25 ml tuberculin syringe. The right testis was treated similarly with an equal volume of saline. To test the specificity of the actinomycin D effect, an identical procedure was followed with streptomycin and puromycin at dosages as high as 10 or 100 times that employed for actinomycin D. After ten days of treatment the animals were sacrificed, the testes were weighed separately on a torsion balance, and testis and epididymis were immersed in Bouin's fixative for routine histologic preparation.

*Results and Discussion.*—Estradiol administration to ovariectomized rats causes proliferation of the endometrium as well as growth and keratinization of the vaginal mucosa. The hormone action begins with a marked increase in RNA content and RNA synthesis rate in the uterus.[6, 8] According to these reports, the latter event precedes the active phase of protein synthesis. In the work now reported, the vaginal mucosa has been selected as the target organ of estradiol action. The inhibition of RNA synthesis has been achieved by local administration of actinomycin D, which has been adequately demonstrated to inhibit DNA-dependent RNA biosynthesis[9–11] in a variety of bacterial and mammalian cells. Actinomycin D (0.5 $\mu$g), applied locally by means of cotton plugs, inhibited completely the development of vaginal cornification (Table 1). The vaginal smear remained of the castrate type throughout the period of observation, and the histologic preparations showed no evidence of cornification. Estrogen-treated animals with saline-saturated vaginal plugs developed cornified vaginal smears on the second or third day of treatment. That the actinomycin D blockade was limited to the vagina is revealed by the uterotropic effect noted in treated animals (Table 1). These observations suggest that the secondary biologic effect of estradiol in causing proliferation and cornification of the vaginal epithelium can be completely blocked as a result of the inhibitory effect of actinomycin D on RNA synthesis in the target tissue.

TABLE 1

INHIBITION OF ESTRADIOL-INDUCED VAGINAL CORNIFICATION BY LOCAL APPLICATION OF ACTINOMYCIN D

| Treatment | | | No. with vaginal cornified within 72 hr | Average Uterine Weight | |
|---|---|---|---|---|---|
| (Subcutaneous) daily | (Intravaginal) topical | Number of animals | | Wet mg | Dry mg |
| Nil | Nil | 6 | 0 | 84 | 17 |
| 0.6 $\mu$g estradiol-17$\beta$ | Saline only | 6 | 6 | 204 | 49 |
| 0.6 $\mu$g estradiol-17$\beta$ | 0.5–5.0 $\mu$g* actinomycin D | 9 | 0 | 195 | 47.5 |

* Cotton plugs saturated with 0.5, 2.0, or 5.0 $\mu$g actinomycin D in saline solution had the identical effect of inhibiting vaginal cornification without preventing uterine stimulation. Three castrate females were employed at each dose level.

Further observations were made utilizing as the hormone-target organ system, the effect of exogenous gonadotrophin (human chorionic gonadotrophin) on the development of the immature rat testis. In gonadotrophin-treated animals, intratesticular injection of doses as low as 0.05 $\mu$g of actinomycin D daily caused a significant inhibition of testicular growth as compared to the contralateral control testis (Table 2). Similar treatment with streptomycin or puromycin did not influence the response of the testis to gonadotrophin. A daily dose of 5.0 $\mu$g puromycin, 100 times the effective dose of actinomycin D, failed to cause a significant difference in weight between the treated and control testes.

TABLE 2

EFFECT OF INTRATESTICULAR INJECTION OF ACTINOMYCIN D, PUROMYCIN, OR STREPTOMYCIN ON TESTICULAR RESPONSE TO CHORIONIC GONADOTROPHIN*

| Antibiotic† | Daily dose ($\mu$g) | Age at start (days) | Final Testis Weight: Right (saline) mg ± S.D.‡ | Final Testis Weight: Left (antibiotic) mg ± S.D. | P value |
|---|---|---|---|---|---|
| Actinomycin D | 0.05 | 35 | 830.8 ± 44.5 | 539.8 ± 63.1 | $P < 0.02$ |
| Actinomycin D | 0.1 | 25 | 410.7 ± 30.8 | 227.2 ± 63.6 | $P < 0.05$ |
| Streptomycin | 0.5 | 21 | 343.3 ± 7.3 | 348.4 ± 2.97 | $P > 0.5$ |
| Puromycin | 0.5 or 1.0 | 25 | 406.5 ± 46.8 | 408.3 ± 62.5 | $P > 0.5$ |

* Chorionic gonadotrophin was administered subcutaneously at a daily dose of 62.5 International Units.
† Six animals were employed in each group.
‡ Standard deviation was calculated by the formula: S.D. = $\sqrt{\frac{\Sigma d^2}{n}}$.

Cross sections of actinomycin D-treated testes revealed a mixed picture with large areas of seminiferous tubules in the immature state, nonpatent, small in diameter (Fig. 1). In other areas of the gland some tubules had enlarged and become patent, and complete spermatogenesis could be seen. Nevertheless, in keeping with the small weight of the gland, immaturity of the tubules was the characteristic histologic finding. The Leydig cells were nonaggregated and gave no evidence of maturation changes. The epididymis contained some spermatozoa, but the predominant feature was the presence of large numbers of sloughed immature germ cells, characteristic of testicular impairment. In contrast, the saline-treated testes from the same animals had responded normally to gonadotrophin treatment. Spermatogenic waves were complete in most tubules, and tubular growth and tortuosity was apparent (Fig. 2). The corresponding epididymis was filled with spermatozoa.

To confirm the specificity of the actinomycin D blockade, gonadotrophin-treated immature males received 0.05 $\mu$g actinomycin D in the left testis and considerably higher doses of puromycin in the right testis. In each case, testicular development proceeded in the puromycin-treated testis, while the actinomycin D-injected gland remained small and immature (Table 3).

TABLE 3

COMPARATIVE EFFECT OF ACTINOMYCIN D AND PUROMYCIN ON TESTICULAR RESPONSE TO CHORIONIC GONADOTROPHIN

| Actinomycin D $\mu$g | Puromycin $\mu$g | Final Testis Weight: Left (actinomycin D) mg ± S.D. | Final Testis Weight: Right (puromycin) mg ± S.D. | Significance |
|---|---|---|---|---|
| 0.05 | 0.5 | 334.7 ± 47.7 | 507 ± 27.2 | $P < 0.01$ |
| 0.05 | 2.0 | 226.8 ± 17.9 | 462 ± 25.8 | $P < 0.01$ |

See footnotes, Table 2.

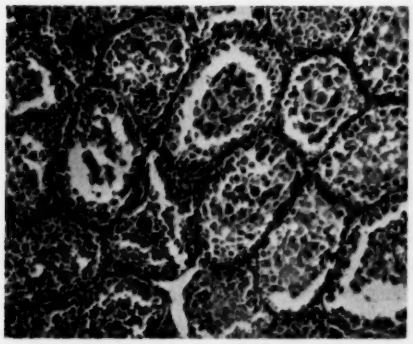

FIG. 1.—Cross section of testis from 45-day-old rat which received 62.5 IU chorionic gonadotrophin for ten days systemically and had concurrent daily intratesticular injections of 0.05 μg actinomycin D. Note immature tubules, incomplete spermatogenesis, and no evidence of gonadotrophin stimulation.

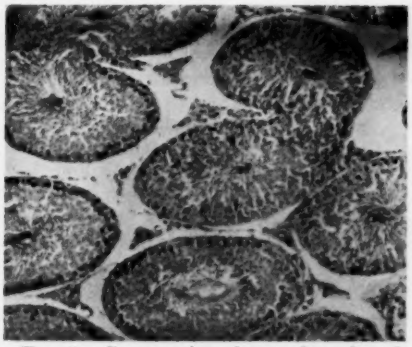

FIG. 2.—Cross section of contralateral testis of animal described in Fig. 1. This gonad received intratesticular injections of saline during the period of gonadotrophin treatment. Note complete spermatogenesis, tubular growth, and indication of normal response to gonadotrophin.

Finally, a group of four 20-day-old males (littermates) was treated with 0.05 μg actinomycin D daily for ten days in the left testis and the equivalent volume of saline in the right testis. It could be expected that puberal levels of endogenous gonadotrophin release and the initiation of puberal changes in the testes would not occur during the treatment period. The animals received no exogenous gonadotrophin. At the completion of the experiment the weight of the saline-treated testes averaged 109 ± 11 mg, and the actinomycin D-treated glands were essentially the same in average weight, 111 ± 9.1 mg. This indicates that the quiescent gonad is not significantly affected by local injection of actinomycin D. Consideration of the total experimental series would suggest that the ability of the testis to respond to gonadotrophin stimulation is blocked.

In view of the recognized interference by actinomycin D in the formation of polyribonucleotides, these results indicate the necessity of initial stimulation of RNA synthesis in order to achieve target organ changes in response to estradiol or chorionic gonadotrophin stimulation. Since similar findings have been reported with respect to the action of growth hormone,[4] thyroxin,[12] and androgen,[13] the possibility is suggested that the unifying mechanism in the action of a variety of stimulatory hormones, irrespective of their chemical nature, is the triggering of messenger RNA synthesis, specific for initiating the secondary biologic events characteristic of the target organ concerned.

*Summary.*—Estradiol-induced vaginal cornification in ovariectomized rats can be prevented by the intravaginal application of actinomycin D. The uterotrophic effect of the systemically administered estrogen is not affected in these animals. Intratesticular injection of actinomycin D prevents the immature rat testis from responding to exogenous gonadotrophin administration, while puromycin and streptomycin do not cause a similar blockade of hormone stimulation. It is suggested that a variety of stimulatory hormones, irrespective of their chemical nature, are characterized by the ability to influence the synthesis of messenger RNA as a prerequisite for the secondary biologic events characteristic of the particular target organ.

The authors are pleased to acknowledge the assistance of C. N. Srinivasan, J. K. Nandi, and G. N. A. Nayar. The studies were carried out with the aid of a grant from the Ford Foundation.

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## *OLD AND NEW RNA IN THE EMBRYOGENESIS OF THE PURPLE SEA URCHIN**

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*Communicated by Robert W. Briggs, June 3, 1963*

The RNA involved in embryogenesis may be stored originally in the unfertilized egg as well as newly synthesized in the course of development. A major portion of the RNA reserve of the sea urchin egg is ribosomal. The ability of these ribosomes to participate in protein synthesis is limited by a lack of messenger RNA (mRNA);[1–3] but after fertilization the rate of ribosomal synthesis of protein increases, presumably because mRNA has been newly synthesized or in some way made available. Chemical measurements indicate that there is practically no change in the amount of RNA present through early development.[4, 5] Nevertheless, incorporation of various radioactive precursors into RNA of sea urchin embryos has been detected.[6, 7] If this incorporation indicates only turnover, then a substantial net synthesis of any one RNA component would have to come at the expense of another. An increase with development in the concentration of one such component, transfer RNA, was deduced previously[3] and has been corroborated by the data to be presented.

In the experiments reported here, the major components of RNA of the sea urchin egg have been characterized by sedimentation analysis, and their fate in the course of development has been examined together with the nature of the RNA newly synthesized at the various embryonic stages. The sedimentation pattern of incorporation following a short exposure (pulse) to radioactive precursor has been compared to that at the end of a subsequent incubation in the

presence of unlabeled precursor (chase). The portion of radioactive RNA of the pulse destined for stable components will be found associated with them following the chase. On the other hand, any rapidly degraded RNA will be detectable only after a pulse and not at the end of a chase. The course of embryogenesis of the sea urchin, it will be demonstrated, is marked by striking changes in the character of pulse and chase RNA ($RNA_p$ and $RNA_c$, respectively).

*Methods.*—Batches of eggs from one to three females of the species *Strongylocentrotus purpuratus* (Pacific Bio-Marine Supply Company, Venice, California) were pooled. Fertilized eggs were allowed to develop[7] at 20°C in artificial sea water[8] in the presence of penicillin (30 mg/ml) and streptomycin (50 mg/ml). At the various developmental stages 1 ml of eggs or embryos were settled by light centrifugation and suspended to 5 ml with sea water for a period of 10 or 20 min, during which time they were exposed to a concentration of either 3.5 or 7 $\mu$M uridine-$H^3$ (2.77 mC/$\mu$mole; New England Nuclear Corporation). At the end of this pulse period the eggs were suspended in sea water containing unlabeled uridine (5 mM). Half of this suspension was centrifuged quickly. The embryos in the pellet were frozen immediately to −80°C and stored. The other half of the suspension was centrifuged lightly, then resuspended in 100 ml of sea water containing 0.5 mM uridine. These embryos continued developing for 4 hr. After this chase period, the embryos were centrifuged, and their pellet was frozen and stored as above.

For further processing, the eggs or embryos were homogenized in a solution of 0.5% sodium dodecyl sulfate (SDS), 1 mM $MgCl_2$, 0.1 *M* NaCl in 0.01 *M* acetate buffer pH 6.0. Samples were immediately submitted to phenol extraction at 60°.[9] Purified RNA[10] in the above solution less SDS was applied to a 5–20% linear sucrose gradient,[11] containing 100 $\mu$g/ml bentonite. Samples were centrifuged for 11 hr at 25,000 rpm in the Spinco head SW 25.1. Equal fractions were collected. Optical densities at 260 m$\mu$ were determined, and the radioactivities of aliquots of the fractions in scintillation fluid[12] were measured in a scintillation counter. Duplicate pulse-chase experiments with different batches of eggs were performed for every embryonic stage represented. All figures have been normalized to a total of 10 $O.D._{260}$ units of RNA per gradient.

The sedimentation constants of the various RNA peaks were estimated by adding to the sucrose gradients a trace of $P^{32}$-labeled RNA, prepared from L-cell fibroblasts (gift of R. Perry), containing components of known sedimentation constants.[13] Using the 18S component of the $P^{32}$-marker RNA as a standard, the approximate S values of the other peaks were derived by the method of Martin and Ames.[14]

*Major RNA components of eggs and embryos:* Sedimentation analysis of the RNA ot the eggs of *S. purpuratus* performed after extraction at 60° in the presence of SDS revealed four major components as determined by absorbancy at 260 m$\mu$. The sedimentation constants of the four peaks of egg RNA from the bottom to the top of the gradient (Fig. 1) were estimated to be 25–28S, 18S, 13S, and 4S, respectively. Phenol extraction at room temperature in the absence of SDS produced only 28S, 18S, and 4S RNA. The first two of these components correspond to the known ribosomal RNA's;[15] the last, to transfer RNA. Phenol extraction of egg ribosomes with SDS at 60° gave the 13S RNA as well as the 28S and 18S material. This RNA component was apparently derived from ribosomal RNA as a breakdown product. The RNA of two other species, *Arbacia punctulata* and *Lytechinus pictus*, were not susceptible in this way to these conditions of extraction.[16] The amount of extracted RNA sedimenting at 13S diminishes considerably in the course of development of *S. purpuratus*, from about 30% in the egg to less than 5% in the pluteus.

Approximately 6% of the total RNA of the egg is 4S. This value rises by a factor of 2 and 3 when the blastula and gastrula stages, respectively, are reached (Fig. 2). A net increase in the concentration of transfer RNA during development had been previously[2] deduced from measurements of polypeptide synthesis by cell-free ribosomal preparations from other species of sea urchin.

*Pulse and chase RNA:* (a) *The unfertilized egg:* Nucleosides are utilized to a lesser degree before than after fertilization;[7] nevertheless, their incorporation into the RNA of the unfertilized egg is detectable. The characteristics of this incorporation were studied by sedimentation analysis of RNA from eggs that had been incubated with $H^3$-uridine for a 20-min pulse and those that had been subsequently incubated in unlabeled uridine for a 4-hr chase, following the 20-min pulse. After the pulse, radioactivity was distributed through the gradient into peaks of approximately 4,

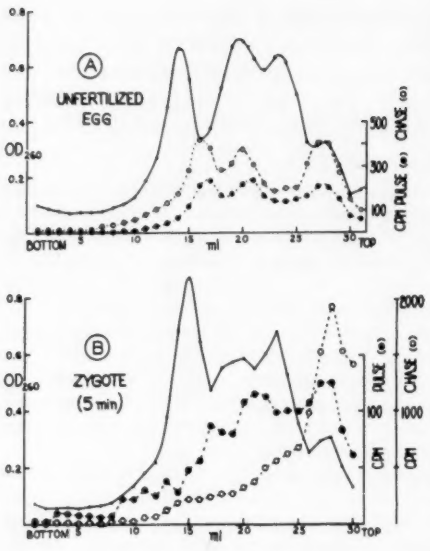


FIG. 1.—Sedimentation diagrams of RNA from (*A*) unfertilized and (*B*) just-fertilized eggs. Eggs were pulsed for 20 min with $H^3$-uridine (7 $\mu$M) and chased for 4 hr in the presence of 0.5 mM unlabeled uridine. Solid curve represents absorbancy at 260 m$\mu$; dotted lines represent radioactivities of $RNA_p$ (●) and $RNA_c$ (○).

17, and 22S (Fig. 1*A*). Following the chase, the same distribution persisted, but with a slight increase in specific activity. The RNA represented by these peaks, apparently, is of a relatively high degree of stability. Since it does not contribute appreciably to the pattern of optical absorbancy, it probably comprises a very small fraction of the total RNA. In both the pulse and chase the 4S RNA was the only major component labeled. The additional incorporation during the chase may have come from nonexchangeable precursor pools that had accumulated during the pulse.[7, 10] A much longer chase would be needed to determine the extent of turnover of the pulse-labeled material.

(b) *The just-fertilized egg:* Exposure of eggs 5 min after fertilization to a 20-min pulse of $H^3$-uridine produced a pattern of incorporation similar to that of the unfertilized egg (Fig. 1*B*). Although qualitatively the same, the incorporation in the $RNA_p$ of the fertilized egg was greater than that of the unfertilized egg. Furthermore, in contrast to the behavior of the unfertilized egg, a 4-hr chase of the fertilized egg resulted in some incorporation into the major non4S RNA components together with a predominant amount in the 4S RNA (Fig. 1*B*). The increase in specific activity during the chase was too great to decide whether or not the $RNA_p$ peaks at 17 and 22S disappeared or were obscured by the pattern of the chase.

(c) *Pregastrula stages:* At the 8- to 16-cell cleavage stage, the radioactive RNA of a 10-min pulse was spread through the sucrose density gradient but with a peak displayed at approximately 10S (Fig. 2*B*). Incorporation during the chase was mainly into the 4S RNA with appreciable labeling of the 13S RNA and smaller activity in the 28 and 18S regions. The same pattern was displayed up to the 24-hr mesenchyme blastula stage (Fig. 2*C*). During development to this stage, the pulse-labeled peak at 10S became progressively broadened toward the heavy region of the gradient, and the degree of utilization of labeled uridine increased. Figure 2*A* represents a 10-min pulse and 4-hr chase following the first division ($1^1/_2$ hr). The character of the $RNA_p$ is intermediate between that of the just-fertilized egg and those in the cleavage and blastula stages; that is, it is apparently a composite containing the 17 and 22S peak as well as the 10S peak.

(d) *Postgastrula stages:* A pronounced change in the characteristics of the $RNA_p$ occurred after gastrulation. In the pulse of the 45-hr gastrula (Fig. 2*D*), the 10S region no longer predominated in amount of incorporation. Instead, the radioactivity was dispersed throughout the gradient with substantial amounts present in regions of greater than 30S. The corresponding $RNA_c$ was also much different. A large amount of incorporation was found for the first time in the 28 and 18S RNA. At this stage the optical absorbancy of the 13S component was reduced from a peak to a small shoulder, and the radioactivity associated with it also described a shoulder. In the prism (60 hr) and in the 72-hr pluteus (Fig. 2*E*) the pattern of incorporation of $RNA_p$ was similar to that seen in the gastrula. After chasing, a large amount of activity was found in the major RNA components.

*Developmental relationships involving 4S and non4S RNA:* The 4S and non4S RNA can be viewed separately by graphically subtracting the activity (the product of the specific activity and total optical density units) of the 4S RNA from that of the total RNA. The resultant non4S

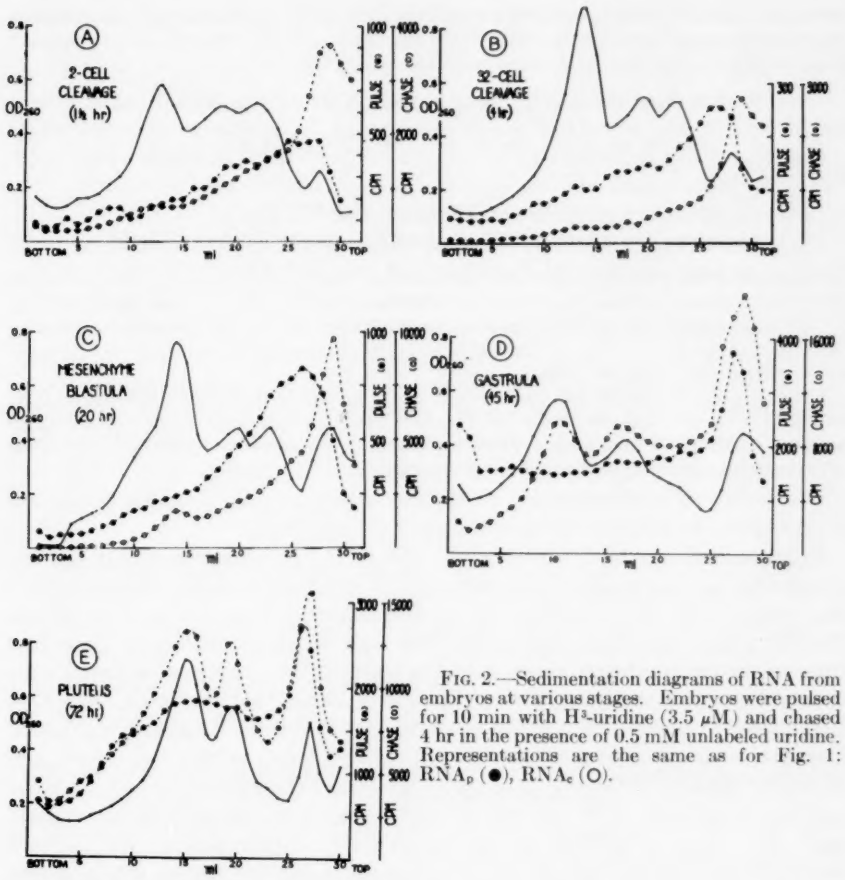


FIG. 2.—Sedimentation diagrams of RNA from embryos at various stages. Embryos were pulsed for 10 min with $H^3$-uridine (3.5 $\mu$M) and chased 4 hr in the presence of 0.5 mM unlabeled uridine. Representations are the same as for Fig. 1: $RNA_p$ (●), $RNA_c$ (○).

RNA's of various stages have been represented on a single scale in Figure 3 against the optical density pattern of the gastrula stage. It is apparent that with development the degree of labeling increases as markedly as the pattern of sedimentation behavior changes. The shift to the heavier region of the gradient is coincident with the pronounced rise in incorporation in ribosomal RNA at gastrulation.

The rapidly synthesized non4S RNA consists of a component ($m$) that is rapidly degraded, concordant with its function as messenger, and a component ($pr$) that is precursor to stable or ribosomal RNA ($s$).[10] The fraction, $pr/(m + pr)$, of the rapidly synthesized RNA ($m + pr$), converted to $s$, can be

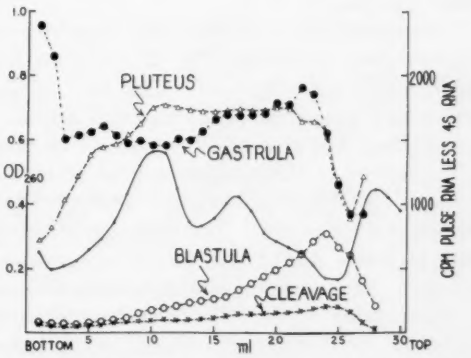


FIG. 3.—A comparison of the sedimentation characteristics on non4S $RNA_p$ at various embryonic stages obtained by graphically subtracting the radioactivity of 4S RNA from the $RNA_p$ of Fig. 2.

estimated for an interval long enough for essentially all of $pr$ to be converted to $s$. An adequate chase represents such an interval. Thus, if the first RNA products (4S RNA + $m$ + $pr$) in a given interval are derived from the same precursor pool, $P$, then

$$P_c / P_p = (m + pr)_c / (m + pr)_p = 4S\ RNA_c / 4S\ RNA_p,$$

$$\text{or } (m + pr)_c = (m + pr)_p\ (4S\ RNA_c / 4S\ RNA_p). \quad (1)$$

If $s = pr$, then

$$pr / (m + pr)_c = s / (m + pr)_p\ (4S\ RNA_c / 4S\ RNA_p)$$
$$= non4S\ RNA_c / (non4S\ RNA_p)\ (4S\ RNA_c / 4S\ RNA_p). \quad (2)$$

Calculations of the fraction of the total radioactivity (cpm) of the rapidly synthesized non4S RNA, as represented in Figure 3, converted to stable non4S RNA yield the values given in Table 1. In the pregastrula stages this fraction was approximately 0.31. The postgastrula stages retained much more of the rapidly synthesized RNA in the ribosomal constituents, about 0.52 in the 45-hr gastrula and 0.59 in the 72-hr pluteus.

While the ratio $s/(m + pr)$ and thus $s/m$ apparently increase in the course of development, the ratio $s$/4S RNA$_c$ also increases (Table 1). This ratio rises from a value of 0.5 in the early cleavage stages to 2.2 in the 72-hr pluteus. Therefore, during development the synthesis of ribosomal RNA increases relative to both messenger and transfer RNA.

*Discussion.*—The development of the sea urchin embryo proceeds in distinct phases, each with a characteristic pattern of RNA synthesis.

*Before and shortly after fertilization:* The RNA$_p$ of unfertilized and just-fertilized eggs has two peaks of approximately 17 and 22S; however, incorporation is somewhat higher after fertilization. In the unfertilized egg the pattern persists through a 4-hr chase indicating a relatively high degree of stability, whereas in a chase of the fertilized eggs incorporation in stable RNA of different sedimentation behavior obscures the pulse pattern, indicating either that the RNA$_p$ has been metabolically degraded or that the synthesis of this type of RNA has diminished. Irrespective of its degree of stability, this RNA$_p$ may be the first messenger, serving to inaugurate the events of embryogenesis. The synthesis of such mRNA could account for the very low but detectable protein synthesis in the unfertilized egg.[2] Its continued production and use after fertilization may satisfy the need for protein synthesis in cell division[17] and account for the rise in this synthesis after fertilization.[1–3, 18]

*Pregastrula stages:* During the early cleavage stages, the nature of the RNA$_p$ experiences a transition (Fig. 2*A*) to a pattern with a predominant peak of approximately 10S (Fig. 2*B*). This may be a transition between the synthesis of messengers patterned by the maternal genome for use before and immediately after fertilization and that of messengers to be involved in the developmental process. This pattern of RNA$_p$ appearing in all of the pregastrula stages exhibits a progressive developmental change, whereby the peak broadens toward the heavier region of the gradient. This peak may comprise a group of mRNA's whose average size increases during this period of development (as the embryo learns longer words?). These messengers may herald differentiation.

*Postgastrula stages:* The final form taken by RNA$_p$ in this progression occurs in all of the postgastrula stages. Its sedimentation pattern is polydisperse with a large proportion of radioactivity of greater than 30S. It is a pattern that has been demonstrated in highly developed growing cells.[9, 10, 19] The sharp rise in ribosomal RNA synthesis, seen in the chase experiments with gastrulae, coupled

with the appearance of substantial radioactivity in $RNA_p$ greater than 30S, agrees with the hypothesis of Scherrer, Latham, and Darnell[19] that this part of the rapidly synthesized RNA is precursor to ribosomal RNA. The calculated proportion of non4S $RNA_p$ that is converted to 28 and 18S RNA rises from less than 30 per cent in the early cleavage stages to greater than 60 per cent in the late pluteus. Thus, the ratio of synthesis of labile or messenger RNA to ribosomal RNA is greater in the pregastrula than in the postgastrula stages. The reservoir of functional ribosomes in the unfertilized egg[1-3] may largely serve the needs of the embryo through the early stages. During this period a greater emphasis may be placed on the synthesis of mRNA needed to program the functional ribosomes already present than on the construction of new ribosomes.

*Summary.*—Distinctly different sedimentation patterns are displayed by the rapidly synthesized RNA of three phases in development: (1) the patterns of the unfertilized and just-fertilized egg have characteristically two peaks (approximately 17 and 22S); (2) a peak of approximately 10S dominates in the pregastrula stages; and (3) the postgastrula pattern is polydisperse. The last is coincident with a markedly enhanced synthesis of ribosomal RNA, as detected by incorporation in chase experiments.

TABLE 1

DEVELOPMENTAL CHANGES IN THE RELATIVE SYNTHESES OF VARIOUS CLASSES OF RNA

| Stage | Hr | Ratio of incorporation (range) $s/(m + pr)$* | Ratio of incorporation (range) $s$/48 RNAe |
|---|---|---|---|
| Cleavage | 1–10 | 0.31 (0.30–0.33) | 0.54 (0.53–0.56) |
| Blastula | 20–28 | 0.34 (0.32–0.38) | 0.87 (0.65–1.10) |
| Gastrula | 45–49 | 0.52 | 1.14 |
| Pluteus | 72–76 | 0.59 (0.55–0.62) | 2.20 (1.9–2.50) |

* Fraction of rapidly synthesized non4S RNA ($m + pr$) converted to stable or ribosomal RNA ($s$ = non4S RNAe).

The author wishes to thank Robert Perry and Jack Schultz for helpful discussions, and Sandra G. Bard and Margaret MacPhee for excellent technical assistance.

* Supported by grant CA 05936-02 from the National Institutes of Health.

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# *EVIDENCE FOR A RING STRUCTURE OF POLYOMA VIRUS DNA**

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*Communicated June 11, 1963*

The DNA of polyoma (PY) virus has two interesting features: it is unusually resistant to heat or formamide denaturation,[1] and it has two distinct components, of sedimentation coefficient 14 and 21, respectively.[2] A two-component sedimentation was formerly shown to be a characteristic of the DNA of papilloma virus,[3] which is considered a member of the same group of viruses.

Evidence presented in this article shows that both these features of polyoma virus DNA are the consequence of special configurational properties.

*Material and Methods.*—Purified polyoma virus was prepared according to Winocour;[4] DNA was extracted from the purified virus with phenol[5] and stored at −70°C. Virus labeled with either $P^{32}$ or $H^3$ was obtained by adding label ($P^{32}$ orthophosphate or $H^3$ thymidine) 24 hr after infecting the cultures. Sedimentation studies were carried out by the band sedimentation method:[6] 3 ml of CsCl solution of density 1.50 were placed in a centrifuge tube and covered with paraffin oil; the sample, of a volume 0.05–0.25 ml. was added dropwise on top of the oil layer. The tube was spun in an SW-39 rotor in a model L Spinco ultracentrifuge at 35 K rpm; in most experiments, spinning time was 4 hr. For shorter runs, the tube containing the CsCl solution and oil was spun for 4 hr before adding the sample, in order to preform the gradient. The CsCl solution was buffered either at pH 7.5 with 0.01 *M* Tris, or at alkaline pH (11.0, 11.8, and 12.5) with 0.01 *M* phosphate buffer.[7] For density gradient equilibrium sedimentation, 3 ml of a CsCl solution of density 1.71 or 1.72 at pH 7.5, and 1.73 at pHs 11–12.5 were used. The solution was covered with oil, spun in the SW-39 head of the model L ultracentrifuge at 29 or 30 K for 70–80 hr. In all cases, fractions were collected from the bottom of the tube. Columns of methylated albumin were prepared according to Mandell and Hershey.[8] Infectivity of polyoma DNA was measured as described by Weil.[5] Pancreatic DNAase Worthington 1x crystallized was used. *E. coli* phosphodiesterase (Lehman enzyme)[9] was a gift of Dr. R. L. Sinsheimer. DNAase and phosphodiesterase digestion were carried out according to Fiers and Sinsheimer.[10]

*Results.*—In brief, the following results were obtained. (1) PY DNA always sediments in two bands, a fast one (*F*) and a slow one (*S*). The *F* and *S* components are stable under a variety of conditions; both are infectious. (2) In the column of methylated albumin the *S* component is eluted at *higher* salt concentration than the *F* component. (3) The following observations were made by density gradient equilibrium centrifugation. At pH 7.5 the bands formed by the *F* and by the *S* component have the same density and do not differ greatly in width; at pH 11.8 most of the *F* component has the density of native DNA, whereas the *S* component has the density of denatured DNA. At pH 12.5 both components are denatured; the *S* component forms a wider band. (4) In band sedimentation at pH 12.5 the *F* component sediments about 2.5 times faster than at pH 7.5; after neutralization and annealing it reacquires the original sedimentation characteristics. The *S* component, on the contrary, migrates at pH 12.5, about the same speed as at pH 7.5. (5) The infectivity of the *F* component is increased several times after treatment at pH 12.5 followed by neutralization; the infectivity of the *S* component is lost after this treatment. (6) Pancreatic DNAase converts the *F* component into the *S* component, with loss of infectivity. The kinetics of conversion is unusual, since it is of first order. These results show that molecules of polyoma virus DNA in different

states exist in the *F* and in the *S* component. The data are consistent with the hypothesis that the *F* component is made up of molecules in ring form, and the *S* component of molecules in linear, or open, form. The results will now be given in greater detail, in terms of this hypothesis.

*Properties of the two components of different sedimentation velocity:* Many preparations of PY DNA, of both small plaque and large plaque type, were examined by band sedimentation in CsCl at pH 7.5. They all contained the *F* and the *S* band. The band pattern observed with this method was found to be similar to that observed with the sucrose gradient method. The major component was the *F* component; it represented 60–80 per cent of the total in DNA preparations which had been extracted only once with phenol, and 90–95 per cent in preparations which had been extracted two or three times.

The ratio of the uncorrected distances traveled by the two components was 1.31 in sucrose gradient with $5 \times 10^{-3}$ *M* $Mg^{++}$, $2.5 \times 10^{-2}$ *M* $K^+$, and $5 \times 10^{-2}$ *M* Tris buffer, pH 7.5; the ratio was 1.22 in CsCl of density 1.50 at pH 7.5.

Both components were infectious and produced plaques of equal type. The average specific infectivity (ratio: infectivity to $OD_{260}$) was two to three times larger in the *F* band (Fig. 1). In the *F* band the infectivity was distributed almost uniformly, whereas in the *S* band infectivity was present only in the frontal part. The *S* band is thus heterogeneous; this band is assumed to consist in its frontal part of infectious linear molecules, obtained by opening the ring, and in its trailing part, of smaller noninfectious fragments.

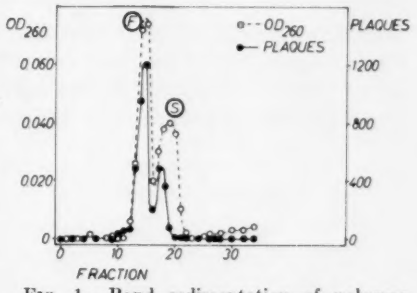


FIG. 1.—Band sedimentation of polyoma DNA at pH 7.5. Polyoma DNA extracted once with phenol and sedimented at 35 K rpm in CsCl of density 1.50 for 4 hr.

*Stability:* Isolated *F* and *S* components were prepared, either unlabeled or labeled with different labels, by collecting fractions corresponding to the peak part of each band. The *F* and *S* components prepared in this way maintained their characteristic sedimentation velocities after prolonged storage at −70°C. Neither conversion from one to the other nor alterations of their sedimentation properties were obtained by the following treatments: exposure to pHs from 4.5 to 11, or to 6 *M* CsCl; treatment with 2 per cent Na-dodecylsulfate for 30 min at room temperature; annealing separately or together at 70°C for up to 1 hr; chromatography in a column of methylated albumin; treatment with the Lehman enzyme.

*Chromatographic properties:* $P^{32}$-labeled isolated *F* and *S* components mixed with unfractionated $H^3$-labeled PY DNA were chromatographed in the column of methylated albumin (Fig. 2). The *F* component was distributed almost uniformly throughout the whole elution band; the *S* component eluted at higher salt concentration. In turn, the frontal part of the elution band was rich in the fast component, whereas the trailing part was richer in the slow component. The infectivity was distributed almost uniformly throughout the chromatographic band.

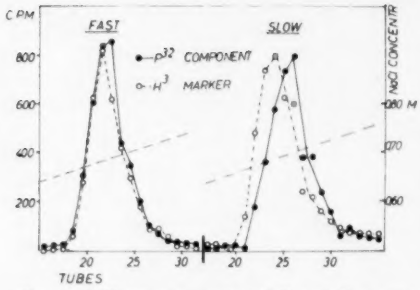


FIG. 2.—Chromatography of *F* and *S* components on a column of methylated albumin. 0.5 μg of DNA was used in each run. A column of 1 cm diameter, 11 cm long was used. The DNA was applied in 0.55 *M* NaCl, 0.05 *M* phosphate buffer pH 6.9. The elution gradient was linear. Fractions of 2 ml each were collected. To each fraction 0.03 ml of a 4% serum albumin solution and trichloroacetic acid to a final concentration of 5% were added in the cold. After centrifugation the pellets were dissolved in $NH_4OH$ and counted.

These results contradict the hypothesis that the *S* band is made up of monomer DNA molecules and the *F* band of end-to-end dimers, as suggested by Watson and Littlefield[3] for the two components of papilloma virus DNA of different sedimentation velocity: the dimers should in fact elute at *higher*, rather than lower, salt concentration. The results are compatible with the ring hypothesis, since the linear molecules may well appear longer to the column than the ring molecules.

*Density gradient equilibrium centrifugation:* Mixtures of *F* and *S* components, labeled with different isotopes, were banded at pHs 7.5, 11, 11.8, and 12.5. At pH 7.5 the two components gave rise to essentially coincident bands at equilibrium (Fig. 3); the band of the *S* component was wider by a factor of 1.2, perhaps owing to the presence, in this component, of fragments of DNA molecules. The *S* component reached equilibrium more slowly than the *F* component. At pH 11 both components had the buoyant density of native PY DNA, although

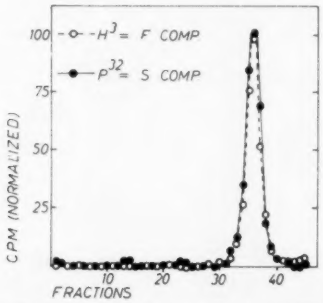


FIG. 3.—Density gradient equilibrium centrifugation of *F* and *S* components at pH 7.5. $H^3$-labeled DNA containing *ca.* 90% *F* component, and $P^{32}$-labeled DNA containing *ca.* 80% *S* component, in amounts of 0.2 μg each, were centrifuged in CsCl of density 1.72 in 0.01 *M* Tris buffer of pH 7.5 for 80 hr at 29 K rpm.

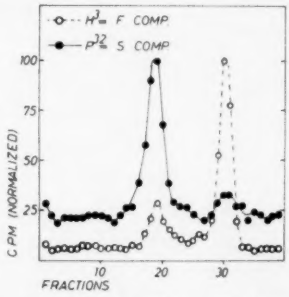


FIG. 4.—Density gradient equilibrium centrifugation of *F* and *S* components at pH 11.8. $H^3$-labeled *F* and $P^{32}$-labeled *S* component in 0.1 *M* phosphate buffer at pH 11.8 were centrifuged in CsCl solution of density 1.73 in 0.01 *M* phosphate buffer, pH 11.8, for 80 hr at 30 K rpm.

the band made up of the *S* component tended to spread toward higher densities, owing to incipient denaturation. At pH 12.5 both components were completely denatured and had a correspondingly higher density;[7] the band of the *S* component was 1.5 times wider than that of the *F* component. At pH 11.8 the *S* component had a denatured density, whereas the *F* component separated into two bands, with

most of the DNA in a band of native density, and the remainder in a band of denatured density (Fig. 4). The proportion of *F* DNA in these bands differed in various preparations.

*Band sedimentation at pH 12.5:* The *S* component sedimented at a speed not very different from that which it had at pH 7.5, whereas the *F* component gave rise to two bands, one (*SF band*) sedimenting like the *S* component at the same pH, the other (*FF band*) sedimenting about 2.5–2.8 times faster (Fig. 5). The proportion of *F* DNA which appeared in the *FF* band was similar to that resisting denaturation in the equilibrium centrifugation. The DNA of the *FF* band reacquired the sedimentation velocity of *F* DNA when annealed at pH 11.8 in 6 *M* CsCl: when rapidly neutralized, most of the *FF* DNA retained the high sedimentation velocity.

The results obtained at alkaline pH are interpreted as follows: the ring molecules (*F* component) are resistant to denaturation, presumably because they do not have free ends; when denatured, they remain topologically unchanged, i.e., still coiled together, and sediment very rapidly, like incompletely denatured DNA.[11] Upon annealing, they reconstitute the ring molecules characteristic of the *F* component. The linear molecules (*S* component) denature more readily, presumably because they have free ends; upon denaturation they give rise to separate single strands, which sediment at a rate similar to that of the native *S* component. The molecules of the *F* component, which sediment slowly at pH 12.5 (*SF* band) and denature readily, are thought to originate from *F* molecules either with single-chain cuts, or with alkali-labile points, such as pre-existing depurinations. Upon denaturation, such molecules would give rise both to single-stranded rings and single-stranded linear chains, which would move close to each other, like the analogous $\phi$X forms.[12]

*Behavior of the infectivity at alkali pH:* Treatment at pH 11.8 or 12.5 abolished the infectivity of the *S* component. The infectivity of the *F* component was not affected by treatment at pH 11.8; after treatment at pH 12.5 and rapid neutralization it was increased about four times. This increase can be probably attributed to increased uptake of partially denatured DNA by the cells.[1]

The two bands produced by the *F* component when sedimented at pH 12.5 were both infectious; in the *SF* band the specific infectivity (ratio: infectivity to radioactivity) was maximal in the frontal and absent in the trailing part of the band.

These results show that the *SF* band is heterogeneous. The heterogeneity is also shown by the different sensitivity to the Lehman enzyme of the various fractions composing the band. The DNA present in the frontal part of the band was resistant to the enzyme, whereas that of the trailing part was partly hydrolyzed. The infectivity was not affected by the enzyme. A possible interpretation of these results is that the infectious DNA present in the frontal part of the *SF* band is made up of single-stranded rings, and the DNA of the trailing part, mostly noninfectious, of single-stranded linear chains. Other interpretations are, however, possible.

*DNAase digestion:* Samples of the labeled isolated components were treated with the enzyme at constant concentration for various lengths of time. Then the sedimentation pattern of each sample was determined, after mixing it with differentially labeled unfractionated PY DNA as marker. *F component* (Fig. 6): As a function of the time of digestion the height of the *F* band decreased, and a band coincident with the native *S* band was formed. The change affected the height of the *F* band but not its position. No component migrating at a velocity intermediate between

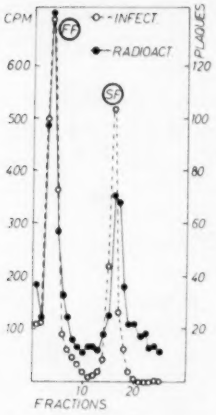


FIG. 5.—Band sedimentation of the *F* component at pH 12.5. 0.2 $\mu$g of $H^3$-labeled PY DNA, made up almost exclusively of *F* component, was mixed with 1.5 $\mu$g of unlabeled isolated *F* component, and brought to pH 12.5 with 0.1 *M* phosphate buffer. The mixture was sedimented at 35 K rpm for two hr in a tube containing CsCl of density 1.50, in 0.01 *M* phosphate buffer, pH 12.5. The tube had been prespun for 4 hr at 35 K rpm in order to preform the gradient. Both radioactivity and infectivity were determined in all fractions.

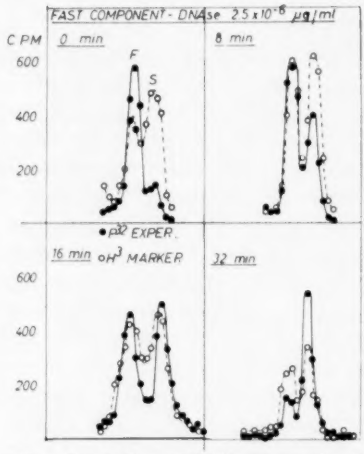


FIG. 6.—Action of DNAase on the *F* component. Isolated $P^{32}$-labeled *F* component DNA (0.5 $\mu$g/ml), unfractionated unlabeled PY DNA (4 $\mu$g/ml), and DNAase, pre-equilibrated at 37°C, were mixed at time zero and incubated at 37°C for various lengths of time. The incubation medium was: 0.04 *M* NaCl, 0.01 *M* Tris buffer, pH 7.5, 0.004 *M* $MgCl_2$, $5 \times 10^{-5}$ gr/ml of bovine serum albumin. DNAase action was interrupted by 0.008 *M* Versene and 0.2 *M* phosphate buffer of pH 9.0. Each sample was then mixed with unfractionated $H^3$-labeled PY DNA, and sedimented at 35 K rpm for 4 hr in CsCl of density 1.50.

those of the *F* and of the *S* band was formed. In the further study of this phenomenon, a DNAase treatment which converts 37 per cent of the *F* component into *S* will be designated as a "converting hit." *S component* (Fig. 7): As a function of the time of digestion the *S* band at first increased in width, and showed subsequently a progressively lower velocity of sedimentation without generating a new separate band.

The results obtained with the *F* component can be interpreted as those obtained by Fiers and Sinsheimer[13] with the DNA of phage $\phi$X 174: that a single scission in the ring molecule converts it to the linear form, thus causing a discrete difference in sedimentation velocity.

*Kinetics of DNAase digestion:* In repeated experiments the kinetics of disappearance of the *F* band as a function of time, at constant DNAase concentration, was found of first order. This result suggested at first that the ring contains an inserted single-stranded segment. To search for such a segment, labeled *F* DNA was exposed to several converting hits with DNAase and then to the Lehman enzyme. No measurable hydrolysis was observed under conditions where 1 per cent hydrol-

ysis could have been detected. We conclude that the DNA of the *F* component does not have a single-stranded segment of sufficient size to explain the kinetics of DNAase action.

In order to interpret the kinetics of $F \rightarrow S$ conversion, the kinetics of DNAase action on either component was studied by sedimenting the enzyme-treated DNA both at pH 7.5 and at pH 12.5, together with untreated DNA, differentially labeled. The changes in sedimentation velocity at pH 7.5 reflect the consequences of *complete scissions* of the DNA molecules; the changes observed at pH 12.5 reflect the consequences of *single-chain scissions*. The following observations were made. *F component:* The effect of DNAase, whether studied at pH 7.5 or 12.5, was similar: at pH 12.5 the *FF* component was converted into the *SF* component at exactly the same rate at which the *F* component was converted into the *S* component at pH 7.5. This result shows that DNAase did not produce single-chain cuts in the ring molecules, which would have resulted in faster disappearance of the *FF* component at pH 12.5, compared to the *F* component at pH 7.5. Thus, the enzyme broke both chains at once. *S component* (either native or produced from the action of DNAase on the *F* component): At either pH the band formed was modified in the same qualitative way by DNAase; the band became wider, and then its average velocity of sedimentation progressively decreased, as digestion proceeded. The effect was, however, much more pronounced at pH 12.5.

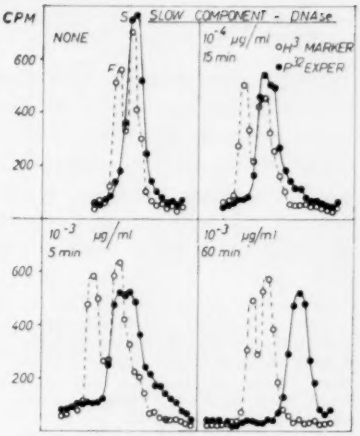


FIG. 7.—DNAase action on the *S* component. Same conditions as in Fig. 6, except that isolated *S* component was used.

To compare the effects observed at the two pHs, the number of scissions was evaluated from the sedimentation behavior of the DNA molecules. When the number of scissions was low, it was evaluated by determining the proportion of material remaining in a position coincident with the *S* band (pH 7.5) or with the *SF* band (pH 12.5). When the number of scissions was higher, it was evaluated from the change in average velocity of sedimentation,[14] with the assumption that the sedimentation velocity is proportional to $MW^{0.37}$ at pH 7.5, and to $MW^{0.5}$ at pH 12.5.[15] In repeated experiments it was found that single-chain cuts, demonstrable at pH 12.5, were produced at a much faster rate than complete scissions, demonstrable at pH 7.5; and furthermore, that complete scissions occurred according to a kinetics which appeared to be of higher order (Fig. 8).

These results show that there is a profound difference in the effect of DNAase on the *F* and on the *S* component.

The interpretation of these results is that the ring molecules are labile for reasons related to their shape. Perhaps the ring form imposes a statistically higher stress on the phosphodiester bonds; hydrolysis of one bond may increase considerably the stress on the complementary bond, making it highly susceptible to hydrolysis by the same enzyme molecule, or to breakage. As soon as the ring opens, the stress disappears.

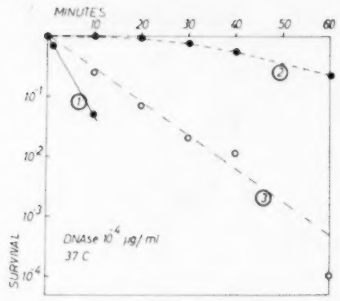


FIG. 8.—Kinetics of DNAase digestion on $H^3$-labeled PY DNA containing almost exclusively *F* component. All rates were determined within a single experiment, by using a single sample which contained DNA, 15 μg/ml and DNAase, $10^{-4}$ μg/ml. Survival = proportion of the respective form that remained unchanged. *Curve 1:* kinetics of disappearance of *F* (pH of sedimentation 7.5) or *FF* (pH of sedimentation 12.5). *Curve 2:* kinetics of degradation of *S* (derived from *F*); pH of sedimentation 7.5. *Curve 3:* kinetics of degradation of *SF;* pH of sedimentation 12.5. In Curves 2 and 3 low survivals were calculated from the average number of scissions per molecule, according to the equation: survival = exp (− average number of scissions).

*Effect of DNAase on infectivity:* These experiments yielded two types of results. The first result is that the infectivity of the DNA of the *F* band decreased as a function of the time of exposure to DNAase at exactly the same rate as the radioactivity. This shows that the infectious molecules have the same properties as the bulk of the DNA molecules. Experiments carried out with pure *F* component showed that not more than 10 per cent of the infectivity lost by the *F* component appeared in the *S* band. Thus, $F \rightarrow S$ conversion by DNAase entails loss of infectivity in most, if not all, molecules.

The other result is that the infectivity of the *S* component was more resistant to the action of DNAase than that of the *F* component. The number of hits in the *S* component determined from infectivity loss corresponded to the average number of complete scissions per molecule, as determined from the sedimentation behavior. With increasing exposure to DNAase, as the radioactivity band increased in width, the residual infectivity remained associated with molecules which sedimented like those of the frontal part of the original *S* band.

These results show that in both the *F* and the *S* components loss of infectivity was caused by complete scissions, whereas single-chain breaks were inconsequential. Single-chain cuts had, however, an effect on infectivity, if DNA which had experienced a few DNAase hits was heated at 100°C for 5 min in 0.15 *M* NaCl. This DNA lost all its residual infectivity, in contrast to DNA not exposed to DNAase which increased its infective titer two or three times after heating.[1]

*Conclusions.*—The results here reported are in agreement with the hypothesis that the DNA of polyoma virus is a ring molecule. Many other possible structures which may account for the existence of two components of different sedimentation velocity are contradicted by the results.

According to the results, the ring should be made up of two complete complementary strands, each one separately closed on itself, plectonemically coiled, and not cross-linked. It is likely that the ends of each polynucleotide chain are connected by a special "linker," as in φX DNA.[13] This is suggested by the presence of infectivity in the native linear molecules, in contrast to its absence in at least most of the linear molecules obtained by DNAase treatment. These results also show that infectivity requires the information of the whole DNA molecule and the integrity of its functional units.

Single-chain breaks, as produced by DNAase in infectious *S* molecules, do not abolish infectivity. Thus, either these breaks are repaired after the DNA enters

the cell, or the reading of the DNA molecules for RNA synthesis or replication is undisturbed by the breaks.

The behavior of infectivity at alkali pH suggests that single-stranded rings are infectious; only further experiments may, however, decide whether this is true.

Two results remain to be explained: (1) the difference in sedimentation velocity of the ring and of the linear molecules is larger than for other ring-shaped molecules, such as those of $\phi$X DNA[12] or $\lambda$ DNA;[16] (2) the ring is unstable, as suggested by the kinetics of DNAase digestion. It can be tentatively suggested that they are the consequence of the combination of double strandedness and of the size of the ring.

We are grateful to Prof. R. L. Sinsheimer for useful discussions. The competent and dedicated assistance of Miss Maureen Muir is gratefully acknowledged.

This work will be supplemented by studies with the analytical ultracentrifuge.[17]

* Aided by grants from the National Institutes of Health, U.S. Public Health Service, the American Cancer Society, and The National Foundation.

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## *THE ROLE OF PROTEIN AND NUCLEIC ACID SYNTHESIS IN THE DEVELOPMENT OF RESPIRATION IN POTATO TUBER SLICES**

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*Communicated by W. Z. Hassid, June 13, 1963*

The "wound respiration" of potato tubers has been under investigation for more than 75 years,[1] and attention has recently been focused on the changes which take place when thin slices of this tissue are maintained aerobically. Within a day after cutting, there are striking quantitative and qualitative changes in the respiration: the rate of oxygen consumption increases approximately fourfold, and the respiration becomes relatively insensitive to carbon monoxide and cyanide.[2] Some of the factors which influence these changes have been investigated,[3] but the under-

lying mechanisms which control them are not well understood. Although potato tuber slices are known to be able to synthesize proteins,[4] no causal relationship between synthesis and the rapid development of the respiration has been established. The experiments described in this paper were designed to answer the following question: does the increase in respiratory rate which takes place during the aerobic incubation of potato tuber slices depend specifically on the synthesis of new proteins and nucleic acids? It has been difficult to answer this question in the past, since specific inhibitors of these processes have not been generally available. Although it has been reported that chloramphenicol, an effective inhibitor of protein synthesis in bacterial systems, can prevent the respiratory increase in potato slices,[5] no evidence that this inhibitor was actually blocking protein synthesis was presented, and the high concentration (1 mg/ml) of chloramphenicol used raised the possibility of a general toxic effect. We have found that chloramphenicol at this concentration causes a marked loss of turgidity in the tissue, and its effect on respiration is not always reproducible. In view of the ambiguity of these results, we have investigated the effects of other specific inhibitors. It is shown here that low concentrations of puromycin and actinomycin D completely prevent the development of the rapid respiration in potato slices by inhibiting protein and nucleic acid synthesis in this tissue.

*Materials and Methods.*—Monthly shipments of potato tubers (*Solanum tuberosum*, variety White Rose) were obtained through the courtesy of Dr. H. Timm, Department of Vegetable Crops, University of California (Davis), and stored at 21°C. Cylinders of tissue were removed from a tuber with a cork borer (1 cm diameter), and 1 mm slices were cut using a hand microtome. The slices were washed for 25 min by stirring in sterilized, deionized water and either used immediately ("fresh" slices) or maintained at 21°C in small (6 × 1.5 cm) Petri dishes. To maintain the slices aerobically, they were placed on a piece of filter paper supported by 5 mm glass beads in such a way that they just broke the surface of the liquid. Each dish contained 11 slices and 8 ml of a solution containing 0.02 *M* phosphate buffer (pH 6.7), $5 \times 10^{-3} M$ $MgCl_2$, and $5 \times 10^{-4} M$ $CaSO_4$; 50 $\mu$g/ml of dihydrostreptomycin sulfate, which has no effect on the respiratory changes, was included to decrease bacterial contamination. All glassware was sterilized between experiments.

To follow protein synthesis, the incorporation of leucine-1-$C^{14}$ into the slices was measured at appropriate intervals. The slices were removed from the radioactive solution and washed in 25 ml of nonradioactive leucine (0.2 mg/ml) for 30 min to remove any readily exchangeable leucine-$C^{14}$. They were then extracted with 70% ethanol for 8 hr in a Soxhlet apparatus, after which they were rinsed briefly with absolute ethanol. The alcohol-insoluble residue was dried at 80°C and counted directly in an automatic gas flow apparatus (Nuclear-Chicago) with an efficiency of approximately 5%. The alcohol-soluble material was counted in a Packard Tri-Carb scintillation spectrometer with an efficiency of 55%. To determine the fate of the label, aliquots of the alcohol-soluble material and the hydrolysate (6 *N* HCl, 100°C for 18 hr) of the insoluble residue were chromatographed on Whatman No. 4 paper using a methanol:$H_2O$:pyridine (80:20:4) solvent mixture, and the chromatogram was scanned with a Vangard strip chart counter. The only radioactive compound that could be identified in these fractions was leucine. When the alcohol-insoluble residue was ground to a powder and treated with ninhydrin,[6] no radioactivity could be detected in the $CO_2$ released.[7] This shows that the carboxyl groups of the radioactive leucine in the insoluble fraction were not free; they were presumably linked in peptide bonds.

Ribonucleic acid synthesis was followed by measuring the incorporation of uracil-2-$C^{14}$ into the slices. The readily exchangeable uracil-$C^{14}$ was first removed by washing for 30 min with 0.16 mg/ml uracil. The procedure used to extract soluble leucine did not remove all the soluble nucleotides, so an alternative procedure was developed. The slices were frozen and extracted with 10–20 ml of 60% boiling ethanol for 10 min; this procedure was repeated 5 times, after which the tissue was extracted twice with absolute ethanol. The radioactivity in the soluble fraction and in the insoluble residue were determined as described above for leucine. To determine the nature of the alco-

hol-soluble radioactive components, this fraction was made up to 95% in ethanol and 0.2 *M* in NaCl and allowed to stand for an hour at 0°C. The precipitate which formed was collected, washed, and counted; it contained less than 1% of the radioactivity in this fraction, suggesting that little, if any, RNA had been extracted. When an aliquot of the soluble fraction was subjected to paper electrophoresis in 0.2 *M* ammonium formate buffer (pH 3.7), four radioactive bands were separated and identified by the strip chart counter. Two of these bands corresponded to uracil and UMP, but the other two, lying between them on the paper, were not identified. The radioactivity which remained in the tissue after the alcohol extraction was almost certainly in RNA, since incorporation into this fraction was completely blocked by actinomycin. To obtain direct evidence that the uracil-$C^{14}$ was incorporated into RNA, it was isolated using a modified phenol method.[8] The RNA was analyzed by the sucrose gradient technique,[9] and the profiles of the ultraviolet-absorbing material and the radioactivity were identical. The RNA obtained from actinomycin-treated tissue contained no radioactivity.

Respiratory rates were measured using standard Warburg manometric techniques. The nitrogen content of the alcohol-insoluble fraction remaining after the Soxhlet extraction was determined by the Kjeldahl method, using red mercuric oxide as the catalyst.

Actinomycin D was a generous gift from Merck, Sharp and Dohme, and chloramphenicol was donated by Parke, Davis and Company.

*Results.*—The general kinetic characteristics of the system under investigation are shown in Figure 1. The respiratory rate of the potato slices changed very little during the first three hr, after which it increased rapidly until it reached a plateau at approximately 15 hr. Our attention will be focused on the 24-hr period during which the rate of respiration increased approximately fivefold. The amount of leucine-$C^{14}$ incorporated into the alcohol-insoluble fraction also increased rapidly after an initial lag period, and evidence that this incorporation corresponds to protein synthesis has already been presented (see *Methods*). Direct measurements of the nitrogen content of the alcohol-insoluble fraction showed that during this period the amount of insoluble nitrogen in the slices increased roughly parallel to the change in leucine incorporation. It seems likely that the amount of leucine incorporation was limited by the amount of radioactive leucine taken up by the slices, since the kinetics of the two processes were very similar (Fig. 2). In any case, the results with leucine-$C^{14}$ indicated that some protein synthesis had taken place within 2 hr after cutting the slices, i.e.,

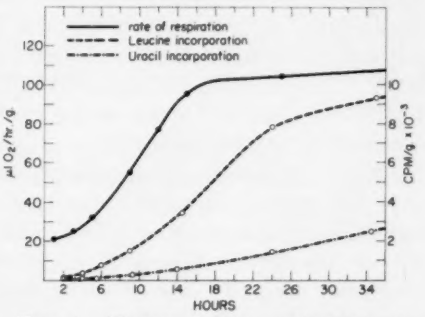


FIG. 1.—Changes in the respiratory rate, leucine incorporation, and uracil incorporation during the aerobic incubation of potato tuber slices. Solutions contained either $0.5 \times 10^6$ dpm leucine-$C^{14}$ or $1.2 \times 10^6$ dpm uracil-$C^{14}$ per 8 ml.

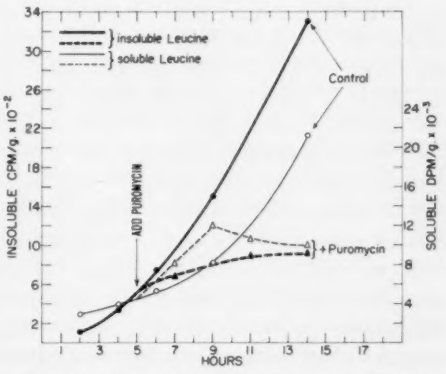


FIG. 2.—The effect of adding puromycin (to $5 \times 10^{-4}$ M) after 5 hr on the uptake and incorporation of leucine ($0.41 \times 10^6$ dpm per 8 ml).

TABLE 1

EFFECTS OF PUROMYCIN ON RESPIRATION AND INCORPORATION

| Puromycin concentration (moles/liter) | Respiratory rate $Q_{O_2}$* | % Inh.† | Leucine incorporation‡ cpm/gm | % Inh. | Uracil incorporation§ cpm/gm | % Inh. |
|---|---|---|---|---|---|---|
| Control | 120 | ... | 9,930 | ... | 1,770 | ... |
| $0.6 \times 10^{-4}$ | 88 | 33 | 6,800 | 32 | 1,440 | 19 |
| $1.0 \times 10^{-4}$ | 77 | 49 | 4,350 | 55 | 1,230 | 31 |
| $2.0 \times 10^{-4}$ | 49 | 76 | 2,770 | 73 | 805 | 55 |
| $4.0 \times 10^{-4}$ | 29 | 97 | 650 | 93 | 356 | 79 |
| $8.0 \times 10^{-4}$ | 25 | 101 | 390 | 96 | 183 | 94 |

* $Q_{O_2}$ = $\mu l O_2$/hr/gm.
† Per cent inhibition of the development of respiration (initial $Q_{O_2}$ = 26 $\mu l$ $O_2$/hr/gm)
‡ $0.51 \times 10^6$ dpm leucine-$C^{14}$ per 8 ml.
§ $1.3 \times 10^6$ dpm uracil-$C^{14}$ per 8 ml.

*before* there was a significant change in the respiratory rate.

The incorporation of uracil-$C^{14}$ into the insoluble fraction started slowly and then increased steadily throughout the experimental period. The slow initial incorporation was not the result of a limitation imposed by the uptake of uracil-$C^{14}$, since the soluble fraction was found to contain 20,000 dpm and 78,000 dpm after 2 and 9 hr, respectively. It is more likely that this lag represented the time required for the conversion of uracil to uridine nucleotides that could be used for the synthesis of RNA. This conclusion is supported by the fact that the ratio of the amounts of UMP-$C^{14}$ to uracil-$C^{14}$ in the soluble fraction increased during the first 9 hr, but decreased in the subsequent 15 hr.

*Effects of inhibitors:* Puromycin is known to be an effective and specific inhibitor of protein synthesis in animal[10] and bacterial[11] cells. Table 1 shows the effects of maintaining potato slices for 24 hr in various concentrations of puromycin: at $4 \times 10^{-4}$ *M*, the increase in respiration and the incorporation of leucine are essentially completely blocked. The effective concentrations of puromycin do not differ greatly from those used with other types of cells. There was a striking similarity in the degree of inhibition of the respiratory rise, on the one hand, and of leucine incorporation, on the other. Uracil incorporation was slightly less sensitive to puromycin than the other two processes, suggesting that the inhibition of RNA synthesis was a secondary effect.

The amount of leucine taken up by the slices during the 24-hr period was markedly inhibited by puromycin. This finding raises the following question: does the puromycin actually inhibit protein synthesis, or does it simply block the uptake of leucine-$C^{14}$ into the cells and thereby prevent leucine-$C^{14}$ incorporation? To test this possibility, $5 \times 10^{-4}$ *M* puromycin was added to the slices *after* they had been maintained in a solution containing leucine-$C^{14}$ for 5 hr, and the radioactivity in both the alcohol-soluble and -insoluble fractions was followed (Fig. 2). In the first few hr following the addition of the inhibitor, incorporation was severely inhibited, but the amount of leucine-$C^{14}$ in the soluble fraction actually increased above the level in the control. This indicates that the primary effect of puromycin was on protein synthesis and not on the uptake of the amino acid. Further support for this conclusion was obtained from the demonstration that the increase in total nitrogen content of the alcohol-insoluble fraction was prevented by incubation of the slices for 24 hr in puromycin. Puromycin had very little effect on the total uptake of uracil-$C^{14}$ or its conversion to UMP-$C^{14}$.

Puromycin appears to be a relatively specific inhibitor without deleterious side effects. It does not inhibit the rate of respiration. Incubation of day-old potato slices for 24 hr with a concentration of puromycin which completely prevented the development of the respiration did not affect the rate of respiration. Puromycin's effect on development is reversible. Freshly cut slices were kept in $5 \times 10^{-4}$ *M* puromycin for 5 hr, removed, washed for 1 hr in distilled water, and then maintained in a puromycin-free solution. At the end of 24 hr (after cutting), the $Q_{O_2}$ of these slices was only 31, but after an additional 24 hr it was 115. The latter rate was the same as that of the day-old controls, indicating that recovery was complete.

Actinomycin D, which inhibits nucleic acid and protein synthesis in bacterial[12] and animal[13,14] cells, completely prevented the development of the respiration in potato slices (Table 2). It also inhibited RNA synthesis completely, at concentrations of actinomycin which are as low as those effective in other systems. Protein synthesis was inhibited, but there was a basal level of leucine incorporation which was not eliminated by the highest concentrations of actinomycin used. When a correction was made for this actinomycin-insensitive leucine incorporation, the degree of inhibition of protein synthesis was found to correspond exactly to the degree of prevention of the respiratory rise. This suggests that only protein synthesis which is dependent on new RNA synthesis is linked to the development of the respiration.

TABLE 2

EFFECTS OF ACTINOMYCIN D ON RESPIRATION AND INCORPORATION

| Actinomycin D concentration ($\mu$g/ml.*) | Respiratory rate $Q_{O_2}$* | % Inh† | Leucine incorporation‡ cpm/gm | % Inh. | Uracil incorporation§ cpm/gm | % Inh. |
|---|---|---|---|---|---|---|
| Control | 111 | ... | 10,680 | ... | 3,070 | ... |
| 2.5 | 74 | 47 | 4,910 | 58 | 1,094 | 64 |
| 5.0 | 54 | 72 | 3,207 | 70 | 580 | 81 |
| 10 | 42 | 87 | 2,297 | 79 | 232 | 92 |
| 25 | 33 | 99 | 1,630 | 84 | 55 | 98 |
| 35 | 34 | 97 | 1,463 | 86 | 49 | 98 |

* $Q_{O_2}$ = $\mu$l $O_2$/hr/gm.
† Per cent inhibition of the development of respiration (initial $Q_{O_2}$ = 32 $\mu$l $O_2$/hr/gm).
‡ $0.69 \times 10^6$ dpm leucine-$C^{14}$ per 8 ml.
§ $1.5 \times 10^6$ dpm uracil-$C^{14}$ per 8 ml.

There was no indication that actinomycin inhibited the uptake of either uracil or leucine, and it did not block nucleotide synthesis. The amounts of uracil and UMP in the alcohol-soluble fraction actually increased in the presence of this inhibitor. Actinomycin itself did not inhibit the respiratory rate at any time; for example, incubation of day-old slices for 24 hr in 25 $\mu$g/ml actinomycin did not affect the respiratory rate, even though RNA synthesis was prevented. This finding does not agree with the suggestion[15] that "the metabolic events which give rise to the respiratory increment must occur continuously for the rise to be sustained." Slices maintained in actinomycin showed no signs of injury and remained turgid throughout the experimental period. In order to determine whether slices which had been kept for a day in actinomycin still retained the characteristics of "fresh" slices, their response to dinitrophenol was studied. After 24 hr in 25 $\mu$g/ml actinomycin, the respiratory rate of the slices was exactly doubled by a brief exposure to $5 \times 10^{-5}$ *M* dinitrophenol (pH 6.0); this stimulation corresponded exactly to the effect on "fresh" slices.[3]

A number of other compounds which have been widely used as inhibitors of protein and nucleic acid synthesis had no effect on the development of the respiration in potato slices, even when used at relatively high concentrations. These included: 5-fluorouracil (100 $\mu$g/ml), 5-fluorodeoxyuridine (200 $\mu$g/ml), 8-azaguanine (150 $\mu$g/ml), 5-methyltryptophan (1.5 mg/ml), and streptomycin (250 $\mu$g/ml). The results obtained with chloramphenicol (1 mg/ml) were varied, but when $MgCl_2$ and $CaSO_4$ were added to the incubation medium, this inhibitor did prevent the respiratory rise without apparent injury.

*Effect of time of inhibitor addition:* In order to clarify the time course and the sequence of the events underlying the respiratory change, inhibitors were added at intervals after the slices had been cut. For these experiments, concentrations of puromycin ($5 \times 10^{-4}$ *M*) and actinomycin (25 $\mu$g/ml) which completely prevented the respiratory rise when added initially were used. The results are shown in Figure 3; the length of time that the slices remained in the inhibitor, i.e., 24 hr minus the time of addition, is indicated on the abscissa. In all cases, the respiratory rates and amounts of radioactivity incorporated were determined 24 hr after the slices were cut.

It is clear from the $Q_{O_2}$ determinations that increases in the respiratory rate were only prevented when the inhibitors were added within 8 hr after cutting the slices. The addition of actinomycin after one hr completely prevented the development. Additions after longer periods, but before 8 hr, resulted in partial inhibitions; for example, when actinomycin was added after $2^1/_2$ hr, the rate of respiration only doubled. On the other hand, when puromycin was added after $2^1/_2$ hr of aging, the development of the respiration was still completely prevented. These results indicate that the RNA synthesized in the first $2^1/_2$ hr after cutting is able to support a doubling of the respiratory rate, presumably by directing the synthesis of specific proteins; but if this protein synthesis is blocked, there is no increase in respiration.

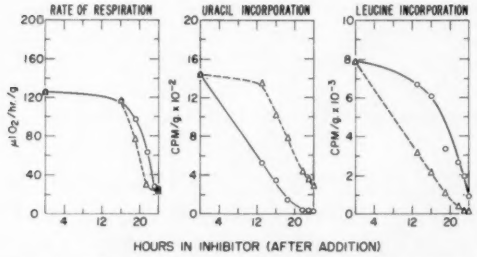


FIG. 3.—The effects of delayed additions of puromycin (- - -) and actinomycin D (——) to final concentrations of $5 \times 10^{-4}$ *M* and 25 $\mu$g/ml, respectively, on the development of respiratory capacity, uracil ($1.2 \times 10^6$ dpm per 8 ml) incorporation, and leucine ($0.41 \times 10^6$ dpm per 8 ml) incorporation. The inhibitors were added after 0, 1, $2^1/_2$, 5, 8, and 11 hr incubation, and all measurements were made at the end of 24 hr.

A comparison of the kinetics of uracil and leucine incorporation with the effects of delayed inhibitor additions (Fig. 3) leads to the conclusion that actinomycin blocked uracil incorporation within an hr after its addition, and puromycin blocked leucine incorporation in less than 2 hr. The differences in the effects of the two inhibitors can be seen most clearly in the experiment where they were added after 11 hr of aging (i.e., 13 hr in inhibitor): actinomycin had little effect on the subsequent leucine incorporation but markedly inhibited the uracil incorporation, and puromycin had exactly the opposite effects. It will be recalled (Table 1) that, when puromycin was added initially, the uracil incorporation was blocked

TABLE 3

EFFECTS OF ADDING PUROMYCIN (TO $5 \times 10^{-4}$ *M*) AND ACTINOMYCIN (TO 25 μG/ML) AFTER 5 HR OF INCUBATION ON RESPIRATION AND INCORPORATION DURING SUBSEQUENT 19 HR

| Process measured | Per cent inhibition by Puromycin | Actinomycin |
|---|---|---|
| Development of respiration | 67 | 38 |
| Leucine incorporation | 93 | 42 |
| Uracil incorporation | 49 | 97 |

more than 90 per cent; this indicates that RNA synthesis is more dependent on protein synthesis during the first few hr than in the later periods. The sensitivities of the respiration and the synthetic activities to the addition of inhibitors after 5 hr of aging are compared directly in Table 3. The per cent inhibitions were calculated on the basis of the changes which would have taken place in the subsequent 19 hr if the inhibitors had not been added. With puromycin, the percentage inhibition decreased in the following order: leucine incorporation, development of respiration, and uracil incorporation; with actinomycin, the sequence was: uracil incorporation, leucine incorporation, and development of respiration. These experiments indicate clearly that the primary effects of actinomycin and puromycin on the potato slices are on nucleic acid and protein synthesis, respectively, and they add further support to the view that the development of respiration depends on protein synthesis, preceded by RNA synthesis.

*Concluding Remarks.*—Previous work has shown that the rapid development of the "wound respiration" in potato tuber slices is entirely dependent on the general aerobic metabolism of the tissue.[3] During this period there are marked increases in the activities of a number of oxidative enzymes.[3, 16, 17] There are also rapid and striking changes in the path of carbon, leading to increased activity of both the pentose cycle[18] and the tricarboxylic acid cycle.[19] Our results indicate that all of these changes are dependent on the synthesis of new RNA and proteins. The control over these processes is probably exerted at the level of the cellular DNA, since this is the site at which actinomycin acts.[20] The nature of the specific trigger mechanism which permits the development of the respiration is not known, but synthesis of nucleic acid and protein must be released from some kind of repression soon after the slices are cut. Laties[21] has proposed that the development of respiration may result from the loss of a volatile inhibitor from thin slices of tissue, and has recently suggested that γ-hydroxy-α-ketoglutarate, an inhibitor of tricarboxylic acid cycle activity, may be involved.[22] Our results indicate that release from some respiratory inhibition will not *per se* account for the observed changes, since the development depends on the synthesis of new proteins.

* This investigation was supported by a grant from the National Science Foundation and by a U.S. Public Health Service fellowship (No. I-F1-GM-19,403-01) from the National Institutes of Health.

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## *AN EARLY EFFECT OF ESTROGEN ON PROTEIN SYNTHESIS**

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*Communicated by H. E. Carter, June 10, 1963*

In studies of the mechanism of action of estrogens, Mueller, Gorski, and Aizawa demonstrated that, when protein synthesis was blocked by puromycin, the increases induced by estrogen in phospholipid and ribonucleic acid synthesis were also blocked.[1] They concluded that uterine responses to estrogen are dependent on protein synthesis and pointed out that the elucidation of the nature of this protein synthesis is critical to understanding the mechanism of action of estrogen. Estrogen may stimulate protein synthesis in general by influencing, directly or indirectly, some component of the protein-synthesizing systems of the cell. This would result in the nonselective increase in synthesis of various types of proteins, including those necessary for further expression of the estrogen response. Another possibility is that estrogen brings about the *de novo* synthesis of a limited number of proteins necessary for the uterine response to estrogen. These proteins might in turn influence various metabolic pathways, including the protein-synthesizing systems themselves. It was impossible to distinguish between these two possibilities previously, because studies of the estrogen effect on protein synthesis were carried out at time periods of 4 hr or greater after estrogen administration when the rate of over-all protein synthesis is markedly stimulated.[1, 2]

In order to ascertain the nature of the protein synthesis stimulated by estrogen, we investigated the effect of estradiol-17$\beta$ on *in vivo* protein synthesis in various cell fractions at 2 and 4 hr after hormone treatment. The relationship between RNA and protein synthesis in rats treated 2 hr with estrogen was studied, utilizing the puromycin inhibition of protein synthesis.

*Experimental Procedure.*—Immature female rats weighing approximately 50 gm were injected intraperitoneally with 5 $\mu$g estradiol-17$\beta$ in 0.5 cc of 0.154 *M* NaCl (saline) at 2 and 4 hr prior to sacrifice. Control animals received 0.5 cc saline intraperitoneally 1 hr prior to sacrifice. Protein synthesis was determined by measuring the incorporation of $H^3$-leucine (2 $\mu$c/rat) or glycine-2-$C^{14}$ (1 $\mu$c or 2 $\mu$c/rat) into protein, and RNA synthesis was determined by measuring the incorporation of $H^3$-cytidine (2 $\mu$c/rat) into RNA. All animals were injected with isotope in 0.5 cc saline 1 hr prior to sacrifice. After sacrifice the uteri were excised and homogenized in 5 ml cold homogenization medium.[3] The homogenates were centrifuged at 800 $\times$ *g* for 10 min. The supernate was decanted from each sample, and the residue resuspended in 2 ml homogenization medium and centrifuged at 800 $\times$ *g* for 10 min. The resulting residue is termed the nuclear fraction. The two supernates for each sample were combined and centrifuged at 15,000 $\times$ *g* for 15 min to obtain the mitochondrial fraction. The supernate was then centrifuged at 105,000 $\times$ *g* for 1 hr to obtain the ribosomal fraction. In certain cases, the supernatant fraction was further centrifuged for 3 hr at 105,000 $\times$ *g* to obtain a postribosomal pellet. The final supernate in all cases is termed the soluble fraction. Five ml cold 5% perchloric acid (PCA) was added to each residue, and 2 ml cold 25% PCA was added to each supernate. After centrifugation, the supernate was discarded, and the protein residue was washed 3 more times with 5 ml of 5% PCA. This was followed by successive washes of 95% ethanol, chloroform and ethanol (2:1), and 2 ether washes to remove lipid. After the last ether wash, the residue was suspended in 1 ml 0.05 *M* Tris buffer (pH 7.4) and 25 $\mu$g ribonuclease was added to liberate the RNA. The suspension was incubated for 2 hr at 37.5°C with occasional stirring. The reaction was then stopped with 1 ml 1.0 *N* HCl, and the samples were centrifuged. The supernate containing the mixture of ribonucleotides and oligoribonucleotides was evaporated to dryness under heat to hydrolyze the oligonucleotides. The residue was chromatographed on paper in an isobutyric acid:0.4 *N* $NH_4OH$ (75:25) solvent system. The cytidine monophosphate (CMP) spots were eluted in 2 ml distilled water and quantitated by their ultraviolet absorption. Aliquots of the samples were counted in a Packard Tri-Carb scintillation counter.

The protein residues were rewashed once with PCA followed by the organic washes described above. The dried residues were then weighed and counted using the scintillation technique.

RNA polymerase in the nuclear fraction was assayed in a system similar to that described by Weiss.[4] Uterine nuclei were incubated 10 min at 37°C with 1 $\mu$c $H^3$-cytidine triphosphate (Schwarz BioResearch) in 1.0 ml of medium at pH 8.0 containing 100 $\mu$m Tris-Cl, 10 $\mu$m cysteine, 5 $\mu$m $MgCl_2$, 75 $\mu$m KCl, 30 $\mu$m NaFl, 1 $\mu$m ATP, 0.4 $\mu$m GTP, 0.4 $\mu$m UTP. At the end of the incubation, cold 5% PCA was added, and the nucleic acid-protein residue was prepared by the washing procedure described above (up to ribonuclease treatment). The dry residue was weighed, dissolved in hydroxide of hyamine, and counted in a scintillation counter. Data were calculated as counts per minute per mg of dry residue.

Animals receiving puromycin injections were injected with 5 mg puromycin dihydrochloride in 0.5 cc saline 15 min before estrogen administration. A second injection of the same amount of puromycin was made 1 hr after the first puromycin injection. Animals not receiving puromycin were injected with a control saline solution.

*Results.*—Figure 1 summarizes data from 3 experiments in which the incorporation of glycine-2-$C^{14}$ into protein of various cell fractions was determined. No significant effect of estrogen on protein synthesis was detectable in nuclear, mitochondrial, ribosomal, or soluble fractions of the rat uterus at 2 hr after estrogen administration. However, 4 hr after estrogen, a marked increase in protein synthesis could be observed in all particulate fractions. The specific activity of the soluble fraction increases only slightly at 4 hr and was no greater than at 2 hr. A comparison was then made between $H^3$-leucine and glycine-2-$C^{14}$ incorporation by injecting the isotopes in combination and assaying the protein at 2 and 4 hr. The data compiled in Table 1 show that both amino acids exhibit the same pattern of incorporation into the cell fractions. Again, the effect of estrogen is virtually lacking at 2 hr, while at 4 hr the specific activities of the proteins of the various

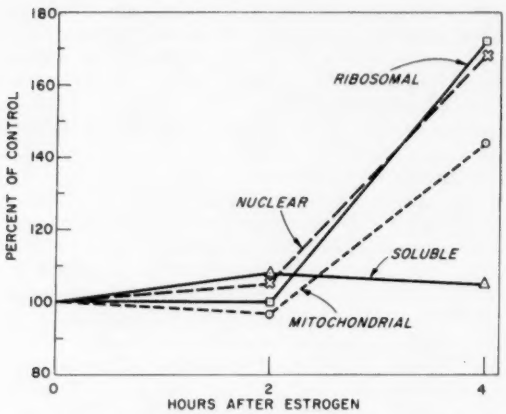


FIG. 1.—Effect of estradiol on protein synthesis in subcellular fractions. 5 $\mu$g estradiol injected intraperitoneally at times indicated. 1 $\mu$c of glycine-2-$C^{14}$ injected 1 hr prior to killing. Each point represents average of 6 groups from 3 separate experiments. Each group used pooled uteri from 6 rats, making a total of 36 rats represented for each time point. Data are expressed as percentage of control specific activities, and methods are as described in text.

cell fractions were increased by estrogen 47 to 239 per cent above control values. In contrast to the previous experiments, the soluble fraction appears to be affected by estrogen at 4 hr. The degree of this response, however, was much smaller than in the particulate fractions.

Since an increase in protein synthesis in response to estrogen could not be detected at 2 hr, it was not considered necessary to separate each particulate fraction in determining the effects of puromycin on protein and RNA synthesis at 2 hr in the experiment shown in Figure 2. When $H^3$-cytidine and glycine-2-$C^{14}$ were injected in combination, no estrogenic stimulation of protein synthesis was observed at 2 hr.

TABLE 1

EFFECT OF ESTRADIOL ON PROTEIN SYNTHESIS IN SUBCELLULAR FRACTIONS OF THE RAT UTERUS

| Cell fraction | Incorporation of Glycine-2-$C^{14}$ into Protein — Hr after estrogen | | | Incorporation of $H^3$-Leucine into Protein — Hr after estrogen | | |
|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 0 | 2 | 4 |
| | Specific activity (DPM/mg) | | | Specific activity (DPM/mg) | | |
| Nuclear | 177 | 171 | 356 | 283 | 223 | 452 |
| | 174 | 176 | 415 | 185 | 244 | 576 |
| Mitochondrial | 349 | 346 | 1,028 | 423 | 573 | 861 |
| | 390 | 334 | 859 | 479 | 443 | 957 |
| Ribosomal | 422 | 616 | 1,359 | 747 | 952 | 1,773 |
| | 542 | 691 | 1,913 | 802 | 976 | 2,031 |
| Postribosomal | 939 | 739 | 1,220 | 1,524 | 1,044 | 1,668 |
| | 782 | 749 | 1,527 | 1,020 | 1,108 | 2,053 |
| Soluble | 705 | 785 | 1,114 | 819 | 1,074 | 1,337 |
| | 708 | 695 | 1,239 | 886 | 903 | 1,594 |

A mixture of 2 $\mu$c glycine-2-$C^{14}$ and 2 $\mu$c $H^3$-leucine was injected intraperitoneally into each rat. Two groups consisting of six animals were used for each time period. The first value listed for each fraction was determined from the same group of animals. Techniques used for fractionation of the cells and the determination of specific activity are described in the text.

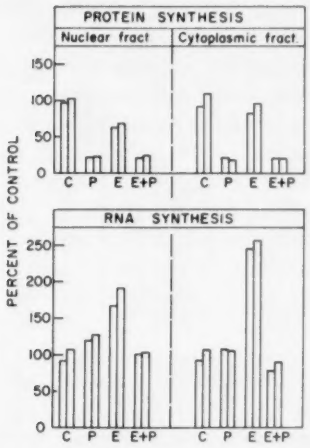


FIG. 2.—Effect of puromycin on synthesis of protein and RNA in uteri from control and 2 hr estrogen-treated rats. Rats received 1 $\mu$c glycine-2-$C^{14}$ and 2 $\mu$c $H^3$-cytidine 1 hr prior to killing. 5 mg puromycin or saline (in controls) was injected hourly, starting 15 min prior to estrogen administration. Nuclear fraction separated as described in text, and supernate designated as cytoplasmic fraction. Each bar represents uteri pooled from 3 rats. Procedures for protein and RNA assay described in text. Data expressed as percentage of control specific activity. C = control; P = control plus puromycin; E = estrogen; E + P = estrogen plus puromycin.

In contrast to the lack of stimulation of protein synthesis at 2 hr, estrogen did increase the synthesis of RNA in both nuclear and cytoplasmic fractions (Fig. 2). Specific activities were considerably higher in nuclear fractions as compared to cytoplasmic fractions, and support previous observations on orthophosphate-$P^{32}$ incorporation into RNA.[1, 5]

Administration of puromycin reduced protein synthesis in both estrogen-treated and nontreated groups to 20 per cent of the control values. At the same time, puromycin blocked the estrogen effect on RNA synthesis at 2 hr in both nuclear and cytoplasmic fractions, while it had no significant effect on RNA synthesis in controls. The results reported here, as well as those from a previous study,[5] indicated that estrogen causes a rapid increase in RNA synthesis. We directed our attention therefore to the nuclear enzyme, RNA polymerase, as a possible rate-limiting step controlled by estrogen.

Figure 3 shows that RNA polymerase had approximately 100 per cent greater activity in the 2 hr estrogen-treated rats as compared to controls. Puromycin blocks the increased activity due to estrogen, but does not have an effect on enzyme activity in the controls. Details of the characterization of uterine RNA polymerase and its reponse to estrogen administration will be reported elsewhere.

*Discussion.*—The results show that at 4 hr the increase in protein synthesis due to estrogen is a generalized response occurring in all cell fractions except possibly the soluble fraction. At this time, apparently the activity of the protein-synthesizing

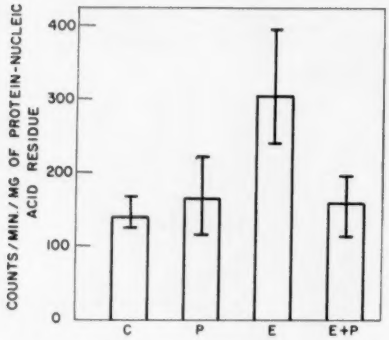


FIG 3.—Effect of estradiol and puromycin on activity of uterine RNA polymerase. Rats injected with 5 $\mu$g estradiol-17$\beta$ 2 hr prior to killing. 5 $\mu$g puromycin administered 15 min prior to estradiol and again 1 hr later. RNA polymerase assay as described under *Experimental Procedure*. Each bar represents average of 4 determinations from 2 nuclear preparations, each representing 3 pooled uteri. Brackets indicate range of 4 values. C = control; P = control plus puromycin; E = estrogen; E + P = estrogen plus puromycin.

systems of all the subcellular organelles has increased due to estrogen, or else the newly synthesized proteins from one type of organelle have migrated throughout the cell, presenting the appearance that all fractions have increased synthetic activity. Recently, similar increases in the accumulation of newly synthesized RNA and phospholipid in all cell fractions within 2 hr after estrogen treatment have been observed in this laboratory.[5] However, as can be seen from the data presented here, there is no detectable estrogen response in protein synthesis at this earlier time. The late appearance of the estrogen response in protein synthesis indicates that the protein synthesis observed at 4 hr could well be dependent on the prior estrogen-stimulated increase in RNA synthesis.

A most important aspect of the results is the lack of a detectable estrogen effect on protein synthesis in general in any fraction at 2 hr, while at the same time, the RNA synthesis stimulated by estrogen is puromycin-sensitive. If we assume that puromycin is only inhibiting protein synthesis, then estrogen must bring about the synthesis of protein necessary for the increase in RNA synthesis.

Demonstration that estrogen causes an increase in RNA polymerase activity which is also blocked by puromycin suggests that this enzyme activity may be responsible for the effects we have observed with respect to RNA synthesis. It cannot be concluded at this time, however, whether estrogen is influencing this activity by directly increasing the rate of synthesis of polymerase or synthesis of some other protein which influences polymerase activity.

Other work in this laboratory[6] has shown that when uniformly labeled $C^{14}$-glucose was incubated with uteri *in vitro*, estrogen significantly stimulated the incorporation of the glucose into protein, RNA, and lipid by 1 hr. Again, this effect was blocked by puromycin.

We are led therefore to the conclusion that one of the earliest estrogen effects is the induction of synthesis of a small group of proteins which are essential for the

elaboration or amplification of initial estrogen action, and that the increased metabolic activity of the uterus due to estrogen action can be attributed to its influence on the production of these proteins. The total number of these proteins must represent such a small percentage of the total protein as not to be detectable by measuring incorporation of labeled amino acids into protein.

It now becomes necessary, in studying the mechanism of action of estrogen, to explain how the interaction of estrogen with a site in the cell results in the selective synthesis of these proteins. Postulations that estrogens function by directly activating the protein-synthesizing systems fail to explain the selective synthesis of proteins and the lag in estrogen effects on protein synthesis that occurs during the first 4 hr. A more likely possibility is that estrogen interacts with a component of the cell which has the capability of controlling the type of protein synthesized. Such a mechanism may be similar to that outlined by Jacob and Monod[7] which supposedly regulates enzyme induction in bacterial systems. However, the role of estrogen in such a system cannot be explicitly defined. While estrogens might act directly as regulators of genetic expression, any hypothesis for estrogen action must take into consideration the possibility that estrogens may function in more remote steps which in turn result in the production of the actual regulators. The difficulty in attacking the estrogen problem has been that the diversity of secondary effects has obscured the initial effects which occur within an extremely short period of time after hormone administration. In view of the apparent estrogen action on specific protein synthesis, further investigations in this area will be necessary to explain the mechanism of estrogen action.

*Summary.*—A marked increase in incorporation of labeled amino acids into proteins of all particulate fractions of the rat uterus occurs 2–4 hr after estrogen administration. No effect on incorporation into portein can be detected at 2 hr. At 2 hr, RNA synthesis and the activity of uterine RNA polymerase are increased, and these increases are blocked when protein synthesis is inhibited by puromycin. It is concluded that the increased synthesis of specific proteins plays an important role in the early action of estrogen and is followed by an increase in the over-all synthesis of protein.

The authors gratefully acknowledge the excellent technical assistance of Mrs. Nira J. Nelson and Mrs. Sylvin Rittschof.

* This investigation was supported by a U.S. Public Health Service grant (A-6327) from the National Institute of Arthritis and Metabolic Diseases. A preliminary report appears as an abstract in *Fed. Proc.*, **22**, 329 (1961).

† Predoctoral Research Fellow of the U.S. Public Health Service.

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# *THE ROLE OF RNA SYNTHESIS IN EARLY ESTROGEN ACTION**

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*Communicated by Henry Lardy, June 10, 1963*

Previous studies from this laboratory have demonstrated that in the rat uterus the early response to estradiol is characterized by a rapid acceleration of synthetic reactions leading to the accumulation of phospholipid, ribonucleic acid, and protein.[1–6] Levels of puromycin which blocked protein synthesis prevented these responses, suggesting that the primary action of this hormone was to accelerate synthesis of rate-limiting enzymes for these anabolic pathways.[7] Since it has been demonstrated in other biological systems that the synthesis of specific proteins depends on the antecedent synthesis of new RNA species,[8–10] it was of interest to inquire whether or not the early estrogenic response required the synthesis of new RNA.

The recent studies of a number of investigators have documented the ability of actinomycin D to block the DNA-dependent synthesis of RNA in cells and in isolated enzyme systems.[11–14] This paper will describe the use of this agent to prevent RNA synthesis in the uterus of the intact rat. It will be shown that under conditions in which the synthesis of new RNA was blocked by actinomycin D, the early acceleration of phospholipid and protein synthesis, as well as a major portion of the imbibition of water resulting from *in vivo* action of estradiol, failed to occur. The requirement for the synthesis of new RNA in the early estrogenic response is discussed.

*Methods.*—Female Holtzman rats of the same age and weighing approximately 180 gm were ovariectomized through the dorsal approach and maintained on a diet of Purina dog chow for at least 3 weeks prior to experimentation. Only rats which were ovariectomized on the same day were used in any one experiment.

The rats were injected intraperitoneally with actinomycin D (375 $\mu$g in 0.5 ml of 0.154 *M* NaCl) or with saline alone as indicated in the figures. At zero hr 10 $\mu$g of estradiol-17$\beta$ in 1.0 ml of buffered saline containing 1% ethanol[6] or the control solution of buffered saline and ethanol was injected via the tail vein. To assess the *in vivo* synthesis of RNA, protein, and phospholipid, a combination of 25 $\mu$c of uridine-$H^3$ (specific activity = 1.0 mc/0.036 mg) and 6 $\mu$c of glycine-$2C^{14}$ (specific activity = 1 mc/12.4 mg) dissolved in 0.5 ml of saline was injected intraperitoneally at 2 and 3 hr. In certain experiments only glycine-$2C^{14}$ was administered.

The rats were killed 4 hr after the injection of hormone by decapitation, and the uteri were removed, stripped of accessory fat, and weighed on a Roller-Smith torsion balance. The uteri were then homogenized in cold distilled water, and the resulting dispersion was treated with 4% perchloric acid (PCA) to remove the acid-soluble fraction. The sedimented tissue residue was washed twice with 4% PCA and then was extracted successively with 90% ethanol, absolute ethanol, and twice with anhydrous ethyl ether. The combined ethanol-ether extracts, making up the crude lipid fraction, were counted in a liquid scintillation counter.

The tissue residue remaining after ethanol-ether extraction was suspended in 0.01 *M* Tris (trishydroxymethylaminomethane) buffer at pH 7.4 and incubated at 37°C for 30 min with 100 $\mu$g of RNAse. The reaction mixture was treated with 4% PCA to obtain the RNAse-released material; this fraction was counted for $H^3$ content in a liquid scintillation counter. The results were expressed as counts per min (CPM) per uterus.

The PCA-insoluble residue was washed successively with 90% ethanol, absolute ethanol, and twice with anhydrous ethyl ether. The residual protein was spread on aluminum planchets with the aid of a small amount of formic acid, and after drying it was counted in a gas flow counter

for $C^{14}$ content. The data on the protein residues are expressed as CPM per mg protein after correction for self-adsorption.

*Results.—The effect of actinomycin D on RNA synthesis in the rat uterus:* In a preliminary experiment two levels of actinomycin D (125 $\mu$g and 375 $\mu$g per rat) were administered to test the ability of this agent to block the *in vivo* incorporation of uridine-$H^3$ into RNA of the rat uterus during the final 2 hr of a 4-hr estrogen treatment. The higher dose inhibited uridine incorporation 90 per cent, whereas the lower dose was slightly less effective. Subsequent trials showed that, within the limits of measurements, the higher dose of actinomycin D blocked RNA synthesis immediately. Since the rats showed no signs of physical difficulty in the 4-hr test period, the 375 $\mu$g dose of actinomycin D was used in all subsequent experiments.

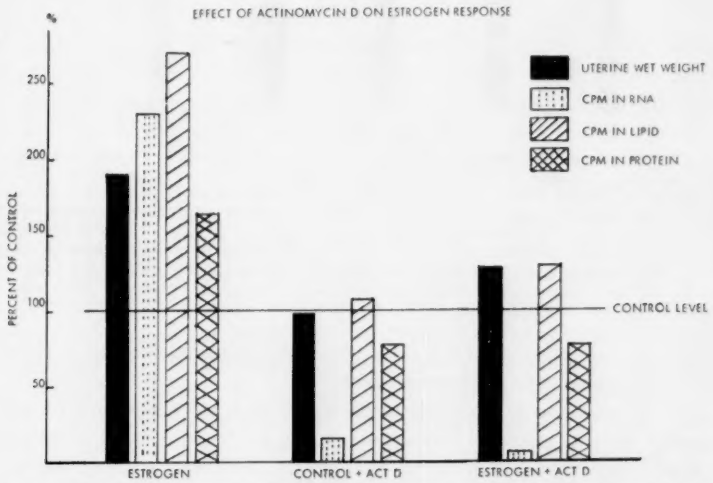


FIG. 1.—Effects of actinomycin D on the early estrogenic response. Groups of 3 rats were injected intraperitoneally with 375 $\mu$g of actinomycin D or control solution 30 min prior to the administration of 10 $\mu$g of estradiol or control solutions. At 2 hr and again 3 hr after the hormone treatment 25 $\mu$c of uridine-$H^3$ and 6 $\mu$c of glycine-$2C^{14}$ were injected intraperitoneally. Four hr after the hormone treatment, the rats were killed and the uteri removed and analyzed for the wet weight, CPM of uridine-$H^3$ incorporated into RNA, and CPM of glycine-$2C^{14}$ incorporated into protein and mixed lipid fractions. Data are expressed as per cent of values obtained for control uteri which were: wet weight = 71 mg; CPM in RNA per uterus = 986; CPM per mg protein = 81; CPM in lipid fraction per uterus = 235.

Figure 1 shows the data from an experiment in which the effects of actinomycin D were studied in both control and estrogen-treated rats; this experiment typified six successive experiments done at different times and with different groups of ovariectomized rats. It is apparent from the data that actinomycin D was equally effective in blocking RNA synthesis in the uteri of both control and estradiol-treated rats; thus, the usual stimulatory effect of estradiol on RNA synthesis was completely eliminated. Experiments described elsewhere had shown that there is a small amount of labeling of RNA in a mammalian cell which is insensitive to the action of actinomycin D. This is confined to the 4S RNA fraction (transfer

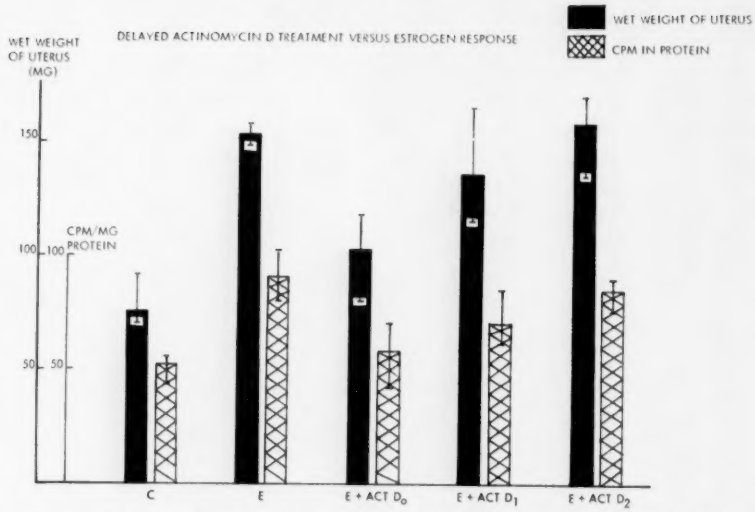


FIG. 2.—Effects of the delayed administration of actinomycin D on the early estrogenic response. Groups of 4 rats each were given intraperitoneal injections of 10 $\mu$g estradiol solution and, where indicated, of 375 $\mu$g of actinomycin D as follows: simultaneously with estradiol administration (E + Act $D_0$); 1 hr after estradiol administration (E + Act $D_1$); and 2 hr after estradiol administration (E + Act $D_2$). At 2 hr and again 3 hr after the hormone treatment, 6 $\mu$c of glycine-$2C^{14}$ was injected intraperitoneally. Four hr after the hormone treatment, the rats were killed, the uteri removed and analyzed for the wet weight, CPM of glycine-$2C^{14}$ incorporated into protein. Data are expressed as mg wet weight/uterus, CPM/mg protein.

RNA) and very likely involves the turnover of the terminal portion of the nucleic acid which accepts an activated amino acid.[14]

*The effect of actinomycin D on the acceleration of protein synthesis by estradiol:* As previously shown, estradiol treatment *in vivo* incites a prompt acceleration of protein synthesis;[6] Figure 1 confirms this effect. The accelerating action of the hormone on protein synthesis is, however, prevented by a level of actinomycin D which simultaneously blocks RNA synthesis. On the other hand, actinomycin D appeared to have little or no effect on the basic process of protein synthesis which is in progress, since it caused only a slight decrease in the incorporation of glycine-$2C^{14}$ into the protein of the control uteri. Accordingly, the acceleration of protein synthesis by the hormone appears to require the synthesis of new RNA.

*The effect of actinomycin D on the acceleration of lipid synthesis by estradiol:* Previous studies demonstrated that estradiol treatment produced a prompt increase in the synthesis of both neutral lipids and phospholipids.[1] In the present experiments the mixed lipid fraction was counted to determine the amount of glycine-$2C^{14}$ which had been incorporated. It had been shown earlier that this incorporation correlated with conversion of glycine to ethanolamine and serine of the phospholipid fraction.[4] Estradiol treatment increased the incorporation of glycine-$2C^{14}$ into the lipid fraction to 270 per cent of the control value (Fig. 1). While treatment with actinomycin D did not significantly affect incorporation of glycine-$2C^{14}$ into the lipid fraction in the control uteri, it prevented approximately 90 per cent of the acceleration caused by estradiol.

*The effect of actinomycin D on the imbibition of water:* A characteristic effect of estradiol on the rat uterus is the imbibition of water during the first 4 hr of treatment (Fig. 1). In the absence of hormone, actinomycin D caused no alteration in the wet weight of the control uteri. In combination with estradiol, actinomycin D limited the imbibition of water to approximately 30 per cent of that obtained with the hormone alone. This fraction of the response was insensitive to actinomycin D, even when this agent was administered 30 min prior to the injection of estradiol.

*The effect of delayed administration of actinomycin D:* In two experiments actinomycin D was administered at different times after the initiation of the hormone treatment to test for any periods of unusual sensitivity. As shown in Figure 2, the administration of actinomycin D 2 hr after the hormone had no effect on the imbibition of water by the uteri in the standard 4-hr test period. Therefore, it appears that the process which supports water imbibition is induced during the first 2 hr of hormone action.

On the other hand, the acceleration of protein synthesis by the action of the hormone was a continuing process requiring an actinomycin D sensitive step. A similar result was found for the estrogenic acceleration of phospholipid synthesis.

*Discussion and Summary.*—A previous report from this laboratory showed that the early estrogenic acceleration of RNA and phospholipid synthesis, as well as the estrogen-induced imbibition of water, could be prevented by levels of puromycin which simultaneously blocked protein synthesis *in vivo*.[7] It was concluded from these data that the early anabolic effects of this hormone depend on protein synthesis, and that this process is the amplifier for the initial hormone interaction with a specific acceptor in the uterine cell.

In the present study, a level of actinomycin D which blocked RNA synthesis effectively *in vivo* also prevented the estrogenic acceleration of phospholipid and protein synthesis. Since the actinomycin D did not affect seriously the basic process of protein synthesis in control uteri during the test period, it would appear that the acceleration of this process as well as the acceleration of lipid synthesis awaited the information contained in the newly synthesized RNA. The observation in these studies that a portion of the water imbibition was insensitive to actinomycin D, yet was sensitive to puromycin,[7] suggests that a part of the information (i.e., RNA) for the hormone response is already available to the protein synthetic machinery of the control uteri, which can, in turn, be activated by the hormone. In accord with this concept, Gorski and associates have recently found that estradiol causes a striking increase of RNA polymerase activity in the nuclei during the first hr of hormone treatment, whereas a number of other anabolic enzymes were found to respond later. The induction of RNA polymerase activity, however, was prevented by puromycin action.[16] In work to be reported elsewhere, it has been shown in centrifugation experiments that estradiol caused an immediate and striking labeling of all classes of RNA.[15]

The summation of these findings prompts the tentative conclusion that estradiol, on engaging a specific receptor system in the uterus, stimulates the synthesis of certain proteins necessary for the induced synthesis of new RNA; the newly synthesized RNA, in turn, supplies the protein-synthesizing machinery with the information essential to the amplification of the initial hormone response.

The authors wish to thank Mrs. Kathleen Deighton for her excellent and cooperative assistance in these experiments.

* This work was supported by grant CA 01897-11 from the U.S. Public Health Service and by funds from the Alexander and Margaret Stewart trust.

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‡ Recipient of a research career award from the U.S. Public Health Service.

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## *THE PARTICULATE NATURE OF NONCHROMOSOMAL GENES IN CHLAMYDOMONAS**

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*Communicated by M. Demerec, June 7, 1963*

The existence of stable genetic determinants not carried on chromosomes and occurring in a wide variety of organisms is well documented and has been summarized in a number of reviews.[1] In most of these studies, the evidence is derived from formal genetic analysis, demonstrating that the pattern of segregation of the mutant phenotype among the progeny of crosses cannot be attributed to chromosomal segregation, whether normal or aberrant. These findings have aroused much speculation, the essence of which can be summarized in terms of two alternative hypotheses: (1) that the mutant phenotypes result from mutations of primary genetic information carried elsewhere than on a chromosome;[1, 2] or (2) that the mutant phenotypes result from metabolic events which shift a series of interlocking reactions from one steady-state level to another, producing an altered phenotype not resulting from any change in primary genetic information.[3]

These alternative views are similar to those that faced Mendel and other analysts of heredity at the turn of the century: Are genetic determinants particulate, discrete units? Do they persist generation after generation whether expressed or not?

The triumph of Mendelian analysis resulted from the recovery of pure parental genotypes among the progeny of $F_1$ hybrids in which genetic factors from the two parents were mixed. Thus, it was shown that alternative factors from the two parents could coexist in the same organism and subsequently segregate out unaltered in later generations. Recessive genes (unexpressed) were no less stable and persistent than their dominant alleles. Mendel dealt with unit factors, one per haploid genome, but his method is applicable as well to factors present in several replicates, provided that segregation occurs.

The finding that streptomycin is mutagenic for nonchromosomal genetic determinants[4] has provided a wide range of new mutant strains for analysis. In this paper we report genetic studies of segregation and recombination with a number of these strains of the alga *Chlamydomonas reinhardi*, demonstrating the particulate nature of the nonchromosomal factors under observation. Our results provide evidence for the existence of a class of genetic determinants which are nonchromosomal in location, stable in replication and transmission whether expressed or not, represented by alternative allelic states in wild-type and mutant cells, and influencing a wide range of cellular traits. It is proposed that this class of genetic determinants be called NC genes.

The nonchromosomal (NC) genes described in this report are transmitted primarily in a uniparental manner in crosses, as previously reported:[5, 6] only one of the two mating types, $mt^+$, regularly transmits its NC genes to all of the progeny, the corresponding NC complement of the $mt^-$ parent usually being lost and not reappearing in subsequent generations. In such a system, the analysis of segregation and recombination would seem to be precluded. However, as previously noted,[7] exceptional zygotes are found in which NC genes from both parents persist. By selecting for these exceptional zygotes it has proved possible to study NC gene segregation and recombination.

*Materials and Methods.*—The parental strains, media, and methods of genetic analysis employed in this study have been previously described, as have the chromosomal gene pairs *act-r/act-s* (actidione-resistance and -sensitivity), *ms-r/ms-s* (methionine sulfoximine-resistance and -sensitivity), $mt^+/mt^-$ (mating type), and the NC gene for streptomycin dependence, *sd*.[6] The new nonchromosomal mutants described in this paper were obtained by growth of a streptomycin-resistant strain, *sr-500*, on streptomycin as previously described.[4] The streptomycin-sensitive strains are denoted *ss*. The three unlinked chromosomal gene pairs (*act-r/act-s*, *ms-r/ms-s*, and $mt^+/mt^-$) segregated in all crosses to be described, and served to identify the four meiotic products (i.e., the zoöspores) and their clonal descendants.

Selection for exceptional zygotes was carried out by plating aliquot suspensions of zygotes on differential media. Gametes of the two parental strains ($mt^+$ and $mt^-$) were mixed under conditions allowing rapid fusion of mating pairs with a zygote yield of about 90%. Zygote suspensions were plated on various media at desired dilutions, and allowed to mature for one week in the dark. Residual vegetative cells were killed by exposure to chloroform vapor for 30 seconds. Germination of zygotes was induced by light. Each resulting colony was derived from a single zygote and contained all of the progeny types, each of which multiplied as a clone within the colony. Progeny analysis was performed by suspending individual zygote colonies in liquid and replating the cells at dilution to give about 200 colonies per plate. Progeny types were identified by replica plating and restreaking when required. Tetrad analysis, the hand-dissection of the four products of meiosis from the germinating zygote, was performed as previously described.[6]

*Selection of exceptional zygotes:* In previous studies[5-7] the survival of the *sr-500* determinant from the $mt^-$ parent in the progeny of up to 10% of the zygotes had been noted in crosses of the type: *ss* $mt^+ \times$ *sr* $mt^-$. This observation raised the possibility that, in some fraction of zygotes in

all crosses, NC genes from both parents might be transmitted to the progeny, thus providing material for an analysis of their segregation patterns.

To explore this possibility, initially we used the NC gene pair *sd/ss*. Exceptional zygotes could be selected in reciprocal crosses, *sd* $mt^+ \times$ *ss* $mt^-$ and *ss* $mt^+ \times$ *sd* $mt^-$, as shown in Table 1. When zygotes of cross 1 (*sd* $mt^+ \times$ *ss* $mt^-$) were plated on minimal medium, thus selecting for the *ss* factor, 0.07% of the zygotes germinated and produced colonies, in contrast to full survival on streptomycin-agar plates. Similarly, in cross 2, full survival occurred in the absence of streptomycin, and 0.08% of the zygotes formed colonies in the presence of the drug. The absence of exceptional zygotes in control crosses 3 and 4, *sd* $\times$ *sd* and *ss* $\times$ *ss*, performed with aliquots of the same suspensions used for crosses 1 and 2, makes it unlikely that mutants in the parental $mt^+$ populations were the source of the exceptions. Further evidence against this possibility is provided by studies of exceptional zygotes in two factor crosses, described below.

TABLE 1

RECOVERY OF ZYGOTE COLONIES IN CROSSES OF STREPTOMYCIN-DEPENDENT AND -SENSITIVE STRAINS

| Cross | Per Cent Zygote Colonies | |
|---|---|---|
| | Streptomycin-agar | Minimal-agar |
| (1) *sd* $mt^+ \times$ *ss* $mt^-$ | 100 | 0.07 |
| (2) *ss* $mt^+ \times$ *sd* $mt^-$ | 0.08 | 100 |
| (3) *sd* $mt^+ \times$ *sd* $mt^-$ | 100 | <0.0001 |
| (4) *ss* $mt^+ \times$ *ss* $mt^-$ | <0.0001 | 100 |

*Progeny of Exceptional Zygotes.*—The genetic constitution of the progeny from exceptional zygote colonies was analyzed by cloning and replica plating. Twenty exceptional zygotes of cross 1 were studied. Both *ss* and *sd* pure clones were recovered from each zygote colony, indicating that both of the parental NC alleles had been transmitted to the progeny.

Presumably, the presence of the *ss* allele made possible germination and colony formation under the selective conditions. As previously described,[6] in *sd* $mt^+ \times$ *ss* $mt^-$ crosses, the *sd* zoöspores from nonexceptional zygotes cannot undergo even one division in the absence of streptomycin. In the exceptional zygotes, however, pure *sd* clones were found after many doublings in the absence of streptomycin in the zygote colonies. The explanation for this behavior, analyzed below, lies in the fact that the segregation of *sd/ss* occurs in the postmeiotic divisions. The four initial haploid products usually contain both *sd* and *ss* factors; these *sd/ss* cells are phenotypically streptomycin-resistant.

The exceptional zygote colonies from cross 1 were found to contain eight genetically different progeny classes; each of the four chromosomal segregant types consist both of *sd* and of *ss* subclones. Thus, *ss* and *sd* must have segregated from each other at postmeiotic divisions. The *ss* clones were in excess because of selection against *sd* in the absence of streptomycin. In cross 2, exceptional zygotes were selected by plating on streptomycin-agar, thus killing any *ss* progeny which might have beer present. As will be shown below, both *ss* and *sd* determinants can be recovered in two-factor crosses, by using the $ac^+/ac^-$ pair to select exceptional zygotes.

Exceptional zygotes have also been selected in crosses involving other NC mutant genes, recently isolated after streptomycin-induced mutagenesis.[4] A series of auxotrophic NC mutants were each crossed to wild type ($mt^-$), and the resulting zygotes plated both on supplemented and on minimal media. The results, listed in Table 2, show the inability of the majority of zygotes in these crosses to produce zygote colonies in the absence of the growth factor required by the $mt^+$ parent.

TABLE 2

RECOVERY OF ZYGOTE COLONIES IN CROSSES OF VARIOUS SM-INDUCED MUTANT STRAINS ($mt^+$) × WILD-TYPE ($mt^-$)

| Mutant strain | Per Cent Zygote Colonies | |
|---|---|---|
| | Supplemented agar | Minimal-agar |
| $ac^-16$ ($ac^-$, chromosomal gene mutant) | 100 | 100 |
| S-3 ($ac^-$) | 100 | 0.10 |
| S-5 ($ac^-$) | 100 | 0.05 |
| S-12 ($ac^-$) | 100 | 0.02 |
| S-22 ($ac^-$) | 100 | 0.05 |
| S-216-1-3 (grows well, then dies on C, poor but viable on M) | + | − |
| S-266-3-5 (auxotrophic: grows on C minus acetate) | + | − |
| S-293-1-1 (auxotrophic: grows on C minus acetate) | + | − |
| S-397-2-1 (auxotrophic: grows on C minus acetate) | + | − |
| S-473 ($ac^-$; tiny on all media) | + | − |
| S-340-1 ($ac^-$; tiny on all media) | + | − |
| S-388-1-3 ($ac^-$; semilethal) | + | − |
| S-408-2-1 ($ac^-$; very leaky) | + | − |
| S-421-1-3 ($ac^-$; nonleaky) | + | − |

C = minimal medium plus 0.1% YE, 0.1% casamino acids, 0.001% each purine and pyrimidine, 0.1% sodium acetate.

This behavior is in contrast to that of chromosomal genes, exemplified by *ac-16*, an acetate-requiring mutant which, in crosses with $ac^+$ $mt^-$, produces zygote colonies equally well in the presence and absence of acetate.

Tetrad analysis of zygotes containing chromosomal as well as NC markers (Table 3), germinated on supplemented media, showed normal segregation of chromosomal gene markers, but all progeny were uniformly auxotrophic like the $mt^+$ parent. Progeny analysis of exceptional zygotes from the same crosses, however, showed the presence of both the wild-type and mutant NC alleles for

TABLE 3

TETRAD AND PROGENY ANALYSIS OF STRAIN S-12 ($ac^-$)

(*A*) Tetrad analysis of unselected zygotes

| Cross | Progeny | No. of zygotes analyzed |
|---|---|---|
| S-12 (*ac⁻ mt⁺ act-r ms-r*) × *ac⁺ mt⁻ act-s ms-s* | all *ac⁻*<br>2 *mt⁺*: 2 *mt⁻*<br>2 *act-r*: 2 *act-s*<br>2 *ms-r*: 2 *ms-s* | 14 |
| 6961a ($F_1$) *ac⁻ mt⁺ act-s ms-r*) × *ac⁺ mt⁻ act-r ms-s* | all *ac⁻*<br>2 *mt⁺*: 2 *mt⁻*<br>2 *act-r*: 2 *act-s*<br>2 *ms-r*: 2 *ms-s* | 14 |

(*B*) Progeny analysis of exceptional zygotes

| Cross | Progeny | No. of zygotes |
|---|---|---|
| 6958c ($F_1$): *ac⁻ mt⁻ act-s ms-r sr-500* × *ac⁺ mt⁺ act-r ms-s ss* | 8 genotypes, each zoöspore clone segregating $ac^+/ac^-$ subclones | 7 |
| | segregating $ac^+/ac^-$, but incomplete sets | 48 |
| *6978a ($F_2$): *ac⁻ mt⁺ act-r ms-s ss* × *ac⁺ mt⁻ act-s ms-r sd* | 16 genotypes, each zoöspore clone segregating $ac^+/ac^-$ and *sd/ss* subclones | 6 |
| | segregating $ac^+/ac^-$ and *sd/ss*, but inplete sets | 7 |
| *6978c ($F_2$): *ac⁻ mt⁻ act-s ms-r ss* × *ac⁺ mt⁺ act-r ms-s sd* | 16 genotypes, each zoöspore clone segregating $ac^+/ac^-$ and *sd/ss* subclones | 25 |
| | segregating as above, but incomplete sets | 29 |

*Exceptional zygotes were selected on streptomycin-agar.

auxotrophy among the progeny, segregating after meiosis as in the *sd/ss* system, and giving rise to eight genetically different progeny classes instead of four.

The complete results of one cross of this type are given in Table 3. Tetrad analysis of 14 unselected zygotes germinated on acetate-agar showed that all of the progeny were uniformly $ac^-$ like the $mt^+$ parent, although they were segregating normally for the unlinked pairs of chromosomal genes, *act-r/act-s* and *ms-r/ms-s*. Similarly, in the $F_1$ backcross generation, ($ac^-mt^+ \times ac^+mt^-$), the progeny were entirely $ac^-$, although the chromosomal markers showed normal segregation.

The zygote colonies recovered by selection of exceptional zygotes were found to contain both $ac^+$ and $ac^-$ clones. Progeny analysis revealed the presence of eight genetically different classes in both the $F_1$ and $F_2$ backcross generations, thus demonstrating the persistent occurrence of postmeiotic segregation of the $ac^+/ac^-$ NC gene pair.

*Segregation and Recombination in Two-factor Crosses.*—Questions raised by the results just cited for one-factor crosses include the following: (1) Are more than one pair of NC alleles preserved in the same exceptional zygotes? (2) Do different pairs of NC alleles segregate independently? (3) At which mitotic division does postmeiotic segregation of NC alleles occur? (4) What segregation ratios are found with NC genes? These questions were investigated with a pair of reciprocal two-factor crosses.

Cross A: *sd* $ac^+mt^+$*act-r ms-s* × *ss* $ac^-mt^-$*act-s ms-r*

Cross B: *ss* $ac^-mt^+$*act-r ms-s* × *sd* $ac^+mt^-$*act-s ms-r*

The $ac^-$ strains used in these crosses were $F_1$'s derived from S-12 (Table 3).

(1) In cross A, one can select exceptional zygotes by plating on acetate-agar without streptomycin, thus selecting for the *ss* NC-allele of the $mt^-$ parent, and permitting equal growth of $ac^+$ and $ac^-$ containing cells. The progeny can then be scored for segregation both of the *sd/ss* pair and of the $ac^+/ac^-$ pair. The *sd* determinant is not lost when *sd/ss* cells are plated in the absence of streptomycin. However, as soon as segregation occurs, the resulting *sd* cells are at a selective disadvantage in the absence of the drug.

In cross B, three different selective media are available: acetate-streptomycin agar, which selects for *sd;* minimal-agar which selects for $ac^+$; and minimal-streptomycin-agar, which selects for both *sd* and $ac^+$. If the zygote colonies, which arise on either type of streptomycin-agar, are transferred as microcolonies to streptomycin-free medium, *ss* clones can be recovered from *sd/ss* cells which had not already segregated.

The fraction of exceptional zygotes arising in these crosses is in the same range (0.02–0.1%) as that found in one-factor crosses (Tables 1 and 2). Furthermore, selecting simultaneously for two NC genes did not reduce the yield to the extent expected if their probabilities of survival in the zygote were independent. All of the zygotes analyzed in these crosses were segregating both for the $ac^+/ac^-$ pair and for the *sd/ss* pair, except for one zygote from cross B selected on minimal-streptomycin-agar which was entirely $ac^+$ though segregating for *sd/ss*.

These data indicate that a fraction of zygotes exists in which NC genes from both parents are preserved. It seems likely that the entire class of NC genes, not only those under observation, survive in the exceptional zygotes and are transmitted

to all the progeny. These results also provide evidence that exceptional zygotes do not arise by mutation of the particular NC genes being studied.

(2) Analysis of progeny from crosses A and B showed that the *sd/ss* and the $ac^+/ac^-$ NC gene pairs segregated independently in postmeiotic divisions. Sixteen genetically different meiotic products were recovered from 25 exceptional zygotes of cross A and 6 of cross B. In addition, 56 incomplete sets were recovered, in which no bias was detected toward any particular recombinant type.

Reciprocal two-factor crosses involving $ac^+/ac^-$ and the NC gene *sr-500* instead of *sd* have also been studied. In the cross $ac^-sr\ mt^+ \times ac^+ss\ mt^-$, zygotes were plated on minimal-agar, and 6 exceptional zygote colonies were recovered. One of them contained all 16 possible recombinant types; the other 5 were incomplete but showed no bias. In the cross $ac^+ss\ mt^+ \times ac^-sr\ mt^-$, selected on streptomycin-agar, all progeny were *sr* as a result of the selection medium, but the 10 zygote colonies analyzed were all segregating for $ac^+/ac^-$ and contained 8 genetically different classes. Here, too, *act-r/act-s* and *ms-r/ms-s* were employed to mark the 4 products of meiosis.

(3) The times of segregation of the NC gene pairs were determined by dissecting exceptional zygote colonies from crosses A and B at early stages after germination and classifying the progeny clones. Cross A was analyzed at the 4–16 cell stage (1–2 doublings of zoöspores) and both crosses were analyzed at the 50–100 cell stage (4–5 doublings). Since the zoöspores do not divide with full synchrony after hatching, the 4 tetratypes are not equally represented in each zygote microcolony. Nonetheless, the data presented in Table 4 were pooled because the segregation of NC genes was found to be unbiased by chromosomal constitution.

TABLE 4

TIME OF SEGREGATION OF NC GENES IN PROGENY OF CROSSES A AND B

| | Type of segregation | | Per cent of progeny of each segregation type | | |
|---|---|---|---|---|---|
| | $ac^+/ac^-$ | *sd/ss* | 4–16 cell (A) | 50–100 cell (A) | 50–100 cell (B) |
| (1) | + | + | 6.0 | 62.0 | 42.0 |
| (2) | + | − | 6.9 | 23.0 | 46.6 |
| (3) | − | + | 2.2 | 6.0 | 2.0 |
| (4) | − | − | 85.0 | 8.8 | 9.4 |
| Number of progeny tested | | | 360 | 272 | 238 |
| Number of zygotes sampled | | | 65 | 12 | 13 |

The results summarized in Table 4 demonstrate that most of the segregation of NC gene pairs occurs in the interval between 2 and 5 doublings of the progeny clones after meiosis. Among the zygote colonies assayed at the 4–16 cell stage, 85% of the progeny had not yet segregated for either NC gene pair, whereas at the 50–100 cell stage, only about 9% of the progeny in both crosses A and B were still of this type. At the later time, in both crosses, the *ac* pair had segregated in over 85% of the progeny. However, the *sd/ss* pair had segregated to a much lesser extent: 68% in cross A and 44% in cross B. Thus, it seems evident that the 2 NC gene pairs segregate, on the average, at different times. It is less clear whether the difference between crosses A and B in segregation time for *sd/ss* is also significant.

(4) The ratio of $ac^+$ and of $ac^-$ clones found among the segregated progeny of crosses A and B was 268 $ac^+$:271 $ac^-$. The two types were recovered with equal frequency in both crosses. The allelic segregation ratios for $ac^+/ac^-$ could be de-

termined with fair accuracy since acetate-medium supports growth at similar rates of both $ac^+$ and $ac^-$ clones. However, no medium could be devised for growth both of *ss* and *sd* clones, and consequently it was not possible to determine segregation ratios for this allelic pair.

*Recovery of New Phenotypes from Crosses.*—In addition to the parental phenotypes which reappear in the majority of progeny, a few new types have been isolated. Extreme $ac^-$ strains have been found among the progeny of cross A, characterized by total inability to grow on minimal medium, a more stringent requirement than that of the parental $ac^-$. A range of new types have been identified by their altered response to streptomycin. Of particular interest is the question whether these new types represent quantitative shifts in the number of determinants, thus showing a dosage effect, or qualitative changes resulting from mutation or recombination. Crosses designed to probe this question are now in progress.

*Discussion.*—In this paper we have described the segregation of nonchromosomal genes by selecting for analysis a class of zygotes in which the nonchromosomal determinants from both parents are transmitted to the progeny. In these exceptional zygotes, the segregation of nonchromosomal determinants occurs after meiosis, among the clonal descendants of each zoöspore (primary meiotic product). By the use of two-factor crosses, in which the segregation of two pairs of nonchromosomal determinants (*sd*/*ss* and $ac^+/ac^-$) could be followed simultaneously, it was demonstrated that (1) both pairs were transmitted in the same exceptional zygotes; (2) the pairs segregated independently, giving rise to 16 genetically different classes of progeny per zygote; (3) on the average, the $ac^+/ac^-$ pairs segregated at an earlier mitotic division than did the *sd*/*ss* pair, over 85 per cent of the progeny having segregated for $ac^+/ac^-$, but only about 50 per cent having segregated for *sd*/*ss* by the 50–100 cell stage (4–5 doublings of the zoöspores); and (4) on the average, the $ac^+/ac^-$ segregation was 1:1 among progeny clones.

*What are exceptional zygotes?* In wild-type *Chlamydomonas*, the mating-type locus appears to control the selective elimination or inactivation of NC genes from the $mt^-$ parent in the zygote at some time between zygote formation and meiosis. Evidence that zygotes from the cross *sd* $mt^- \times$ *ss* $mt^+$ become sensitive to streptomycin before meiosis[6] indicates that the loss (or inactivation) occurs well before meiosis. We have recently found that a heat treatment immediately after zygote formation can increase the yield of exceptional zygotes about 10-fold, suggesting that the fate of NC genes is under enzymic control. Presumably, the exceptional zygotes represent a class in which this control system has failed.

The occasional appearance of streptomycin-resistant progeny in crosses of *ss* $mt^+ \times$ *sr* $mt^-$ was noted in the original description of this system[5] and in subsequent reports.[6, 7] Further analysis was postponed until, with the availability of more NC mutants, segregation could be investigated in reciprocal two-factor crosses. In the present study, the frequency of exceptional zygotes recovered by selective techniques, was in the range of 0.02–0.1%. In our earlier work, we observed frequencies of 4–10 per cent, and recently Gillham,[8] studying the same *ss* $mt^+ \times$ *sr* $mt^-$ cross, has reported frequencies of 3–13%. Perhaps the *sr* determinant is less sensitive to mating-type inactivation than are *ac* or *sd*. These preliminary results suggest that the mechanism of NC gene elimination in the zygote may be amenable to analysis.

*Postmeiotic segregation:* The stability of the NC phenotypes during vegetative growth[5, 6] suggests that a mechanism exists which ensures continuity of NC genes in replication and transmission. The requisite regularity could result if each NC factor were present in many replicates, distributed at random in cell division, or in a few replicates, distributed in an orderly fashion. In our present experiments, the segregation of $ac^+/ac^-$ in the first few postmeiotic divisions suggests that the number of segregating units is low. Gillham's preliminary observations of *sr/ss* segregation[8] also suggest that few units are involved. This evidence would not preclude the existence of many replicates, but does indicate that they would have to segregate as a group (analogous, perhaps, to a multistranded chromosome). One might ask why postmeiotic segregation occurs at all. Perhaps the haploid cells cannot cope with the doubled number of NC genes.

The picture is less clear with respect to the *sd/ss* pair, which segregates on the average later than does the *ac* pair. Perhaps there are more segregating replicates of this NC gene than of the others. We are attempting to clarify this question with crosses in which several different NC mutant genes, including $ac^-$, *sd*, and *sr*, are segregating simultaneously.

*Conclusion.*—Our findings provide strong evidence that the nonchromosomal determinants are carriers of primary genetic information. The alternative hypothesis, that nonchromosomal heredity merely reflects the existence of various cytoplasmic states which alter the expression of chromosomal genes,[3] has been excluded, so far as analysis at the cellular level permits, by the following lines of evidence: (1) postmeiotic segregation of NC gene pairs in clones wich are already haploid for the chromosome complement; (2) retention of identity of NC alleles while present together in the diploid zygote and in the haploid zoöspore; (3) survival of *ss* and $ac^-$ determinants in cells which are phenotypically streptomycin-resistant and acetate-independent; (4) stability of NC mutant genes in vegetative growth and in meiosis.

Thus, the NC genes share with chromosomal genes the properties of stability, mutability, maintenance of identity in heterozygotes, segregation of alleles, and the classical dichotomy between genotype and phenotype. It is hoped that, with this conceptual clarification and with the availability of many new NC mutant strains, the pressing questions of molecular identification and functional role of NC genes will be open to fruitful analysis.

*Summary.*—A method has been developed for analysis of segregation and recombination of nonchromosomal (NC) genes in crosses. As shown in one-factor crosses involving several different NC mutant genes, and in reciprocal two-factor crosses with the gene pairs $ac^+/ac^-$ and *sd/ss*, the NC genes segregate in postmeiotic divisions. The NC mutant genes are considered to be particulate carriers of primary genetic information.

* Supported by grant E-1605 of the U.S. Public Health Service and institutional grant IN-4 of the American Cancer Society.

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## *ASSOCIATION BETWEEN THE SPINDLE APPARATUS AND REOVIRUS**

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*Communicated by George E. Palade, May 31, 1963*

Reoviruses form a common antigenic group of icosahedral particles which measure approximately 750 Å and contain RNA.[1–3] The host range of reoviruses among mammals and mammalian cultured cells is extensive and includes L strain mouse cells.[4] Reoviruses appeared to possess the unique, hitherto unexplained property of aggregating within large inclusions which develop as a continuous band around the nucleus.[2, 5] Within such inclusions, Tournier and Plissier[6] demonstrated the presence of long hollow structures similar to the tubules of the mitotic apparatus. Spindle tubules are present frequently in the vicinity of nuclei, and grouped around the centrioles of cells at interphase. This study was undertaken to test the possibility that infection by reovirus elicits the formation of perinuclear inclusions because this virus develops in association with the spindle.

*Materials and Methods.—Procedures for infecting cells, sampling, and virus assays:* The source of the strain $L_2$ cells and of reovirus type 3 (Dearing strain) and the techniques for the propagation of both have been described previously.[3, 7] Before use, virus lysates were frozen and thawed at least five times to release cell-associated particles. Partial synchrony of infection was obtained by mixing $5 \times 10^6 - 10^7$ cells/ml with virus suspensions at an input multiplicity of 4–8 PFU per cell, and by agitating the mixture continuously for 2 hr at 37° to promote rapid adsorption. After removing unadsorbed virus by two cycles of washing and low-speed centrifugation, the infected cells were diluted to a concentration of $2 \times 10^5$/ml with warm nutrient medium and allowed to incubate in suspension. At desired intervals samples were removed for virus assays, determination of the mitotic index, and electron microscopy. Concentration of infectious particles was assayed as in Gomatos *et al.*[3]

*The mitotic index:* Approximately $10^6$ cells were removed in 5 ml samples, centrifuged into pellets at 800 *g* for 2 min, and resuspended in 0.1 ml of nutrient medium. One droplet of the thick cell suspension was mixed on a microscope slide with a droplet of stain solution (2% aceto-orcein in 40% acetic acid) and covered by a cover slip. The ensuing intense staining of chromatin facilitated the counting of cells in mitosis, in interphase, or with abnormal chromatin configurations.

*Electron microscopy:* Pellets containing $3–5 \times 10^6$ cells were fixed for 3 min in freshly prepared solution of 1% glutaraldehyde (cf. Sabatini *et al.*[8]) in PBS of Dulbecco and Vogt,[9] rinsed three times with PBS, postfixed for 15 min in buffered $OsO_4$,[10] dehydrated, and finally embedded in epoxy-resin mixtures.[11] Contrast in thinly sectioned cells was increased by staining first with a 1% aqueous solution of uranyl acetate (40 min) and then with solution B (10 min).[12]

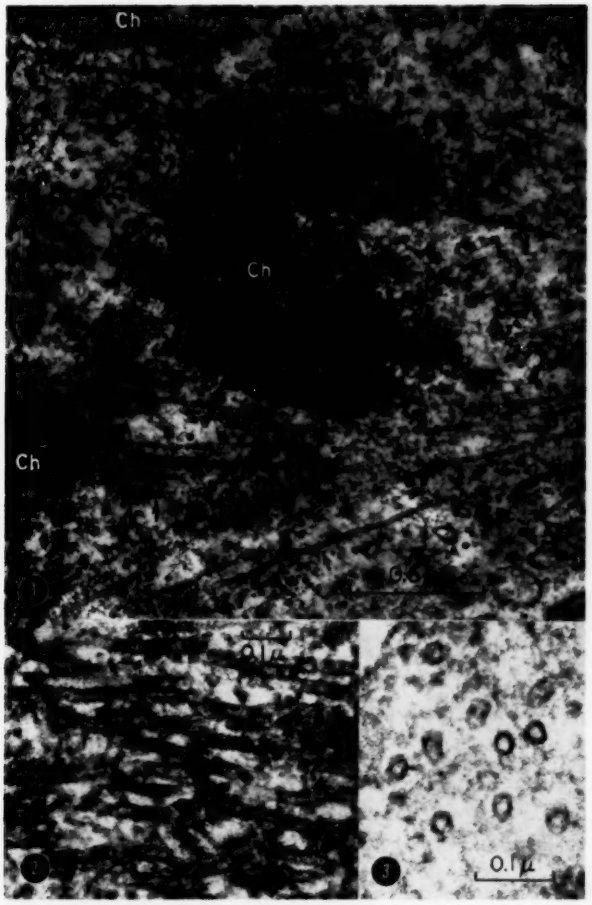



FIG. 1.—Chromosomes (*Ch*) and spindle tubules (indicated by arrows) in a cell in metaphase, sampled from a control culture ×35,000.
FIG. 2.—Group of spindle tubules in the cytoplasm of a cell at late telophase ×100,000.
FIG. 3.—Cross sections through spindle tubules present in a cell at mitosis ×120,000.

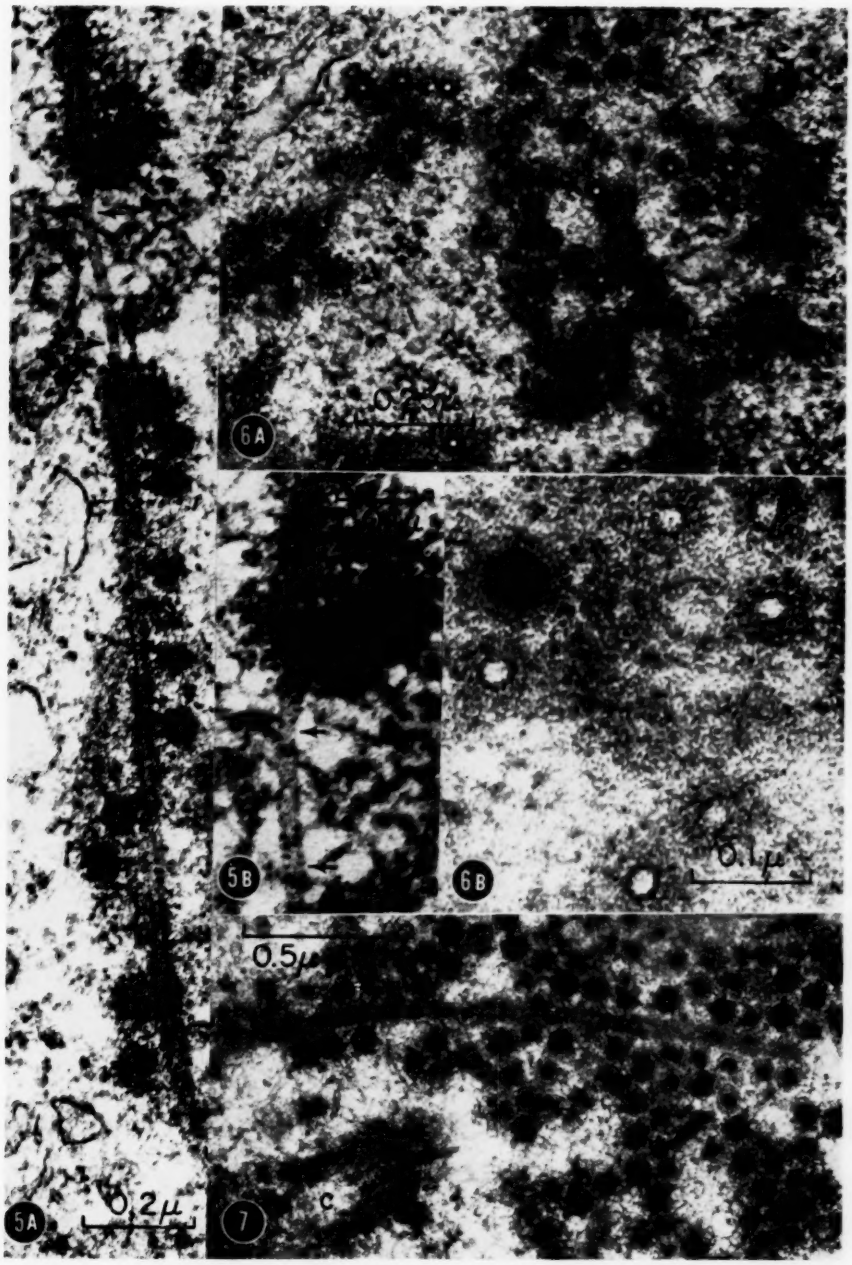
*Results.*—As defined by Mazia and Dan,[13] the mitotic apparatus consists of chromatic and achromatic components. The chromosomes belong to the former, while the centrioles, spindle, and asters are in the latter category. The chromatic component of the mitotic apparatus in L cells was described previously.[14] The corresponding achromatic components have essentially the same morphology as those of other animal cells.[15, 16] A distinctive feature, evident after the double-fixation method used here, was the presence of long and hollow spindle tubules, present in both dividing and interphase L cells (Figs. 1–3). The dimensions of these tubules were: outside diameter 180–230Å, dense walls 30–40 Å thick, inner diameter 160–190 Å, length indeterminate. Some tubules were continuous within the plane of section for almost 3 $\mu$. Tubules observed coursing through the cytoplasm of interphase cells were single, generally straight but occasionally bent in a wide arc, and

*Figures 4–7 represent cells sampled 12 hr after inoculation.*

FIG. 4.—Section through the spindle and chromosomes of a cell in mitosis. Masses of dense material and aggregates of virus particles are closely associated with the tubules of the spindle (indicated by arrows). The large clump of chromatin, evident at the bottom of this field, appears to have resulted from the merger of several chromosomes ×15,000.

randomly oriented with respect to one another. In cells undergoing division, the spindle tubules were arranged in bundles (Figs. 1–3). Centrioles present in the centrosphere zone at interphase, or at the poles during mitosis, were in each case a focal point of assembly for the tubules which nevertheless did not come into direct contact with the walls of centrioles. In cells at mitosis, the tubules traversed the space between the chromosomes and the polarized centrioles, and also formed interconnections between poles. In the zone of the asters, spindle tubules radiated from centrioles and terminated in the cytoplasm at a distance of about 1 $\mu$.

It was ascertained from a preliminary survey that the morphological features of interest arising from infection of L cells with reovirus occurred frequently and could be discerned clearly 12 hr after inoculation. For this reason, samples taken at this particular time were intensively studied. Neither the disposition nor the fine structure of spindle tubules appeared to be affected by the infection. In many




FIGS. 5*A* and 5*B*.—A single spindle tubule in a cell at interphase. Several virus particles lie embedded within the dense fibrous material which coats the tubule along most of its length. The segment of the tubule still uncoated is indicated by arrows ×90,000 and ×160,000.

FIG. 6*A*.—Oblique and cross sections through spindle tubules in a cell at metaphase. Note the coat of dense material around each tubule and several closely associated virus particles ×75,000.

FIG. 6*B*.—Small area of the above at a higher magnification. A single virus particle possessing a dense core is present at the upper left of the field ×180,000.

FIG. 7.—Portion of the cytoplasm of a cell at interphase showing a single, coated tubule and numerous virus particles, one of which lies in close proximity to the centriole (C) ×55,000.

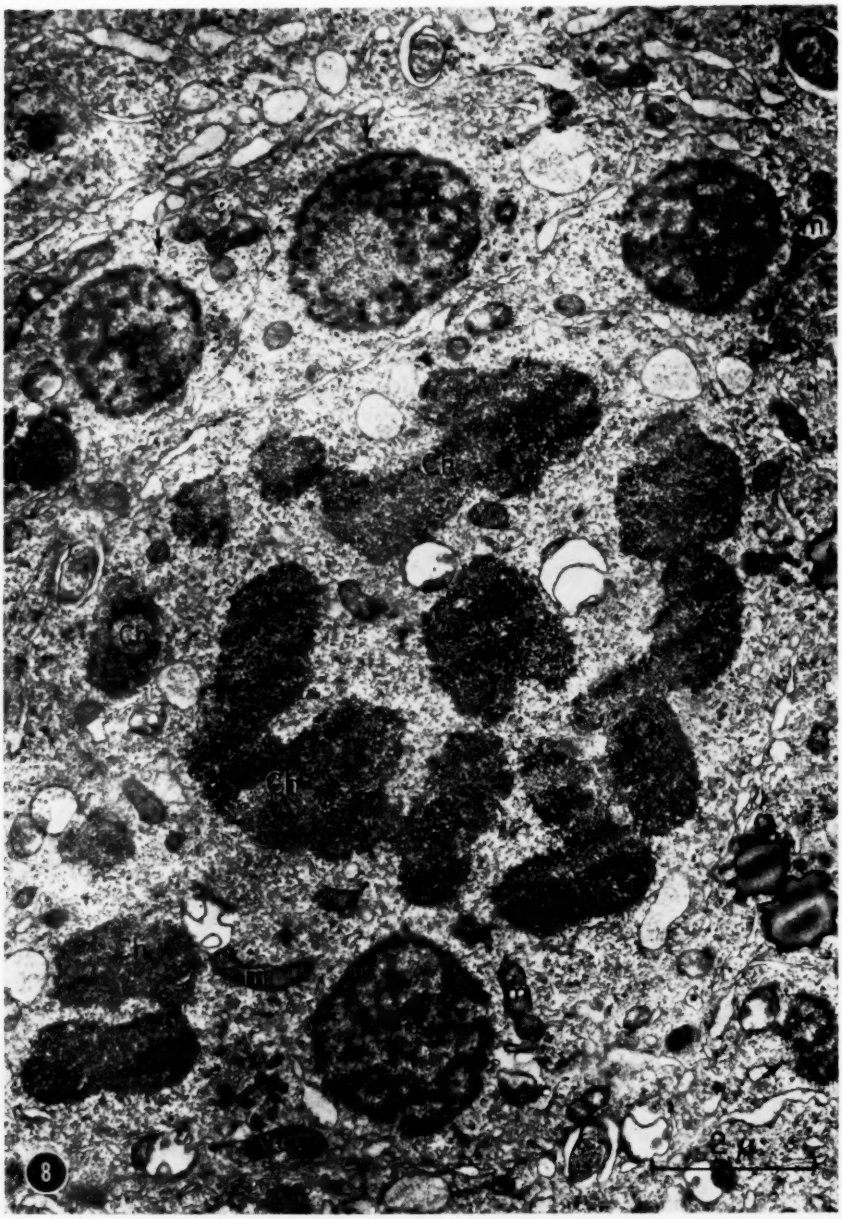



FIG. 8.—A cell at metaphase from a colchicine-treated culture, 12 hr after infection. Separate foci of developing virus particles are indicated by arrows. *Ch:* chromosome; *f:* lipid droplet; *m:* mitochondrion; *ve:* vesicle ×13,000.

instances, however, the tubules were either partially or completely surrounded by a coat of dense material (Figs. 5*A*, 5*B*, and 7), and virus particles were often found either embedded in, or in close apposition to, this coating substance. In extreme cases, almost the entire mitotic spindle was permeated by the dense substance and associated virus (Figs. 4 and 6). When examined at higher resolution, especially in oblique and cross sections of tubules, the dense coat was about 200 Å thick (Figs. 6*A* and 6*B*) and consisted of closely packed, fine, coiled filaments and granules (or cross sections of filaments) with a maximum diameter of about 25 Å.

Both at interphase and during division, inclusions containing virus particles were not restricted to the vicinity of the spindle apparatus but were scattered throughout the cytoplasm. In the centrosphere region they consisted of spindle tubules, coating substance, and aggregates of virus particles, and appeared dispersed and irregular in outline. In the rest of the cytoplasm, the inclusions were more discrete and compact, contained dense substance with interspersed virus aggregates, and only occasionally segments of spindle tubules.

*Although frequent and characteristic, the association with spindle tubules did not appear obligatory for virus development.* This was tested experimentally by using a mitotic inhibitor, namely, colchicine (Eli Lilly Co.) which arrests division at metaphase. Exposure to colchicine ( $1 \times 10^{-7}$ W/V) for 6–8 hr resulted in the disappearance of spindle tubules in dividing and interphase L cells. Viral inclusions continued to develop but only compact, intracytoplasmic forms were encountered (Fig. 8). The results were the same when the drug was added to the cultures 8 hr prior to infection or at the time of virus inoculation. In both cases the inhibitor was maintained throughout virus development.

*Morphological observations suggested that about equal numbers of virus particles were present in the untreated and colchicine-treated cells.* This was supported by assays of infectious virus, as shown in the table. Decrease in titer at 2–6 hr postinoculation showed that virus was still in eclipse at 6 hr. At 12 hr there was a 40- to 90-fold increase in titer, equal in amount in the presence and absence of colchicine.

| Hr after inoculation | Experiment 1 | | Experiment 2 | | Experiment 3 | |
|---|---|---|---|---|---|---|
| | | Colchicine present | | Colchicine present | | Colchicine present |
| 2 | $2.2 \times 10^5$ | $2.2 \times 10^5$ | $2.5 \times 10^5$ | $2.6 \times 10^5$ | $3.4 \times 10^5$ | $3.9 \times 10^5$ |
| 6 | $1.1 \times 10^5$ | $1.1 \times 10^5$ | $2.0 \times 10^5$ | $2.2 \times 10^5$ | $3.0 \times 10^5$ | $3.3 \times 10^5$ |
| 12 | $4.7 \times 10^6$ | $3.9 \times 10^6$ | $1.9 \times 10^7$ | $1.9 \times 10^7$ | $1.3 \times 10^7$ | $1.6 \times 10^7$ |

*The preferential association of reovirus with spindle tubules does not appear to be directly responsible for certain mitotic disturbances observed during virus multiplication.* The mitotic index was determined at intervals following infection to obtain information about the comparative rates of division in the infected and uninfected cultures. As shown in Figure 9, the mitotic rate dropped after manipulation of the cultures. In the controls it gradually rose to a plateau in 10 hr, whereas in infected cultures it rose abruptly by 6 hr and reached a maximum, threefold higher than in the controls at the termination of the 12-hr experiment. It should be emphasized that certain cells from the infected culture possessed highly abnormal mitotic figures. In some of these the chromatin had become very condensed into an amorphous mass (pycnosis). In most instances, however, the abnormal mitotic figures consisted of several clumps of chromatin arranged into rosettes, as if the

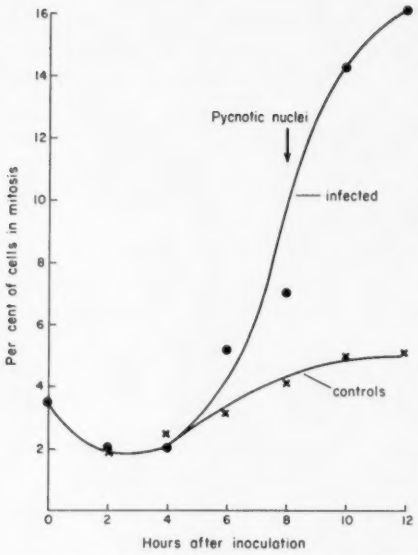



FIG. 9.—Mitotic index in control cultures and reovirus-inoculated cultures.

chromosomes had become partially fused into larger aggregates. These chromatin aggregates, designated as pycnotic nuclei, were included in the mitotic index in Figure 9. They were first observed at 8 hr after inoculation with reovirus and constituted one fifth of all the cells in mitosis. By 10 hr 70 per cent of all mitoses in the infected culture were abnormal and by 12 hr 90 per cent. Images equivalent to those observed by light microscopy were present also in thinly sectioned cells observed in the electron microscope. An example showing apparent fusion of several chromosomes is shown in Figure 4. When colchicine was added to the cultures at the time of infection, there was no observed difference in either the time of appearance or in the number of pycnotic nuclei.

*Discussion.*—The direct association between reovirus and spindle tubules in L cells, reported here, might explain why this virus aggregates in extensive perinuclear inclusions late in its cycle of development in sedentary cells;[2, 3, 5, 6] in such cells spindle tubules are also situated around the nucleus. However, ability of reovirus to multiply in sites other than those where tubules occur and in cells in which tubules have been destroyed by colchicine shows that a direct association between the virus and spindle tubules is not obligatory. In such cases, pools of unidentified precursor material of the spindle might still exist and provide sites for development of reovirus.

The fuzzy dense material coating spindle tubules has not been described previously (cf. ref. 6) and appears to be connected directly with the infection. The nature of this coating substance, whose thickness (200 Å) is comparable to that of the viral capsid, is unknown and must await identification by immunochemical procedures at the electron microscopic level. A recent study using light microscopy has demonstrated the presence of reovirus antigen in the general region of the mitotic apparatus in mammalian cells.[17, 18] Unfortunately, differentiation by light microscopy between antigen of the viral capsid and any which might occur in the tubule-coating substance is not possible. Similarity in dimensions and density of the finely textured material in the coat and viral capsid indicates that viral protein may be present in the fuzzy coating substance.

Increase of the mitotic index above that of the controls 6 hr after inoculation suggests that initially L cells harboring reovirus still in its eclipse phase are stimulated to enter mitosis more rapidly than uninfected cells. Appearance of abnormal mitoses in progressively increasing numbers 10–12 hr after inoculation indicates, however, that during rapid multiplication of reovirus L cells are unable to complete successfully the mitotic sequence. Although it is tempting to ascribe this

malfunction of the divisive process to a "parasitism" of the mitotic apparatus, the failure of colchicine to prevent development of aberrant chromatin configurations suggests that other factors, as yet undiscovered, are responsible for this nuclear pycnosis.

*Summary.*—Reovirus becomes closely associated with the mitotic apparatus of L cells. During virus development the spindle tubules become coated by a dense granular or fibrous material and are contiguous to aggregates of progeny virus particles. Abolition of spindle tubules by colchicine alters the site but not the rate of virus development, showing that the spindle is not obligatory for multiplication of reovirus.

The author is indebted to Professor G. E. Palade for his interest in this work and valuable advice.

* Supported by the Health Research Council of the City of New York (contract U-1141).

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## *BIOSYNTHESIS OF THYROGLOBULIN: RELATIONSHIP TO RNA-TEMPLATE AND PRECURSOR PROTEIN**,†

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*Communicated by Fritz Lipmann, June 26, 1963*

With the use of the antibiotic actinomycin, which inhibits cellular RNA synthesis[1–4] by binding with guanine residues on the DNA,[5] experiments on the physiological activity of the RNA-template for protein synthesis have become possible. Such studies in *B. subtilis* have been interpreted as providing evidence for the short life of the RNA template.[6] In several other systems, however, protein synthesis has been shown to continue in the absence of synthesis of RNA. Protein synthesis takes place in reticulocytes in the absence of RNA synthesis.[7–8]

Protein continues to be made for some time in mouse fibroblasts[1] and fertilized sea urchin eggs[9] after complete inhibition of RNA synthesis of actinomycin. This demonstration of the dissociation between protein and RNA formation has not been limited to animal tissues.[10, 11] These results have led to the concept that the protein-forming units vary considerably in their stability and that, especially in well-differentiated cells, such units may possess long lives.

The thyroid gland is composed of highly differentiated nucleated cells which produce thyroglobulin, a large protein readily isolated for direct study. It appeared likely that the RNA-template specifically concerned with thyroglobulin synthesis would possess considerable stability and that thyroglobulin formation would be resistant to the action of actinomycin. This report will present evidence in favor of this concept and also data on the involvement of a precursor protein in thyroglobulin biosynthesis.

*Experimental.—Thyroid gland slices and incubation conditions:* Fresh lamb thyroids were obtained from a local abattoir and immersed in iced phosphate-buffered saline within 20 min of slaughter. The glands were cleared of fat and connective tissue, sliced thin with a razor blade, and incubated in sterile Eagle's medium for suspension cultures[12] containing 0.1 mM of the non-essential amino acids, penicillin, and streptomycin. Unlabeled leucine was omitted from the original medium and added with or without its radioactive label at the desired specific activity. Incubation volumes of either 2.0 ml or 4.0 ml contained 2–4 slices of thyroid. Each experiment consisted of slices taken from one lobe of the same gland if possible; if not, an equal number of slices from each lobe were added to each flask. Before addition of the slices the flask was pre-incubated for 15 min at 37° in a Dubnoff metabolic shaker into which 100% $O_2$ was bubbled. Gentle shaking and oxygenation were continued for the duration of the experiment. On long incubations, contamination by microorganisms was ruled out by standard bacteriological techniques.

*Preparation of thyroid RNA for sucrose gradient centrifugation:* Following incubation, thyroid RNA was isolated by the method of Hiatt,[13] except that after treatment with DNAase and precipitation with 67% alcohol, the RNA was passed through a 1 × 7 cm G-25 Sephadex column, which removed all traces of phenol, oligodeoxyribonucleotides, and unincorporated labeled precursor. The effluent containing the RNA was reprecipitated with alcohol, taken up in 0.20 ml of 0.10 *M* NaCl and 0.01 *M* potassium acetate buffer pH 5.0, and layered on a 5–20% sucrose gradient containing the same salt and buffer concentrations.

*Preparation of thyroid soluble protein for sucrose gradient centrifugation:* Following incubation, the flask contents were ground in a Potter-Elvehjem glass homogenizer and centrifuged ten min at 27,000 × *g* at 1–2°C. The particulate fraction was extracted with TBS and assayed for radioactivity by the method of Wool and Krahl.[14] The protein in the supernatant fluid was precipitated by the addition of saturated ammonium sulfate at pH 5.0 or pH 6.9 to the desired concentration. The precipitate was separated by centrifugation and dissolved in 4.0 ml of TBS and reprecipitated two times with ammonium sulfate. The final precipitate was dissolved in TBS in the minimal volume that would float on 10% sucrose. A 0.2 ml aliquot was layered on the 10–25% sucrose gradient for centrifugation.

*Sucrose gradient fractionation:* Sucrose gradients were made by the method of Martin and Ames,[15] with 4.7 ml in each tube. Gradients for RNA measurements were 5–20% sucrose with 0.10 *M* NaCl and 0.01 *M* potassium acetate pH 5.0. Gradients for protein measurements were made with 10–25% sucrose in TBS. The Spinco model L preparative ultracentrifuge rotor SW 39 was used for centrifugation at 39,000 × *g*. Protein samples were centrifuged for 9 hr. Ten drop fractions were collected in all experiments, each tube was diluted with 1.0 ml water, the optical density was measured at 260 mμ for RNA and 280 mμ for protein, and an aliquot added to 15 ml of Bray's solution.[16] The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer.

*Thyroglobulin antibodies:* Anti-sheep thyroglobulin antibodies were prepared using rabbits with sheep thyroglobulin purified by the procedure of Derrien, Michel, and Roche.[17] Sucrose

gradient fractions were precipitated with antibody after 3 mg of carrier sheep thyroglobulin had been added. The precipitates were dissolved in 1.0 ml Hyamine 10 × and counted in the scintillation spectrometer. The counting fluid contained 0.4% PPO and 0.01% POPOP in 20 ml of toluene.

*Thyroid autoradiograms:* Incubated slices were fixed in 10% buffered formalin, embedded, and 4 micron slices placed on slides. Kodak AR-10 stripping film was used. Exposure time was six weeks.

*Chemicals:* $H^3$-thymidine (6.05 c/mmole), $H^3$-uridine (1.0 c/mmole), $C^{14}$-uridine (125 $\mu$c/mg), and $C^{14}$-L-leucine (850 $\mu$c/mg) were obtained from Schwarz BioResearch, Inc. $H^3$-L-leucine-4, 5 T (7.1 c/mmole) was from Nuclear-Chicago Corporation. Actinomycin $C_1$ (D) was a gift of Dr. E. Alpert of Merck, Sharp and Dohme Research Laboratories. Carrier-free $NaI^{125}$ and $Pi^{32}$ were from Volk Radiochemical Company. Puromycin was obtained from Nutritional Biochemicals Corporation. Sephadex G25 and G200 were from Pharmacia Fine Chemicals, Inc.

*Results and Comments—Formation of a rapidly labeled RNA in thyroid slices and inhibition of RNA synthesis with actinomycin:* As has been shown for actively multiplying mammalian cells in culture[18–20] and regenerating rat liver,[13] thyroid slices incubated for 15 min with $H^3$-uridine or $Pi^{32}$ incorporate these labeled materials into RNA with a sedimentation constant of about 44S (Fig. 1*A*). Similarly, after longer exposures the label corresponds in position with the 30, 18, and 4S optical density peaks (Fig. 1*B*). Autoradiographic studies with $H^3$-uridine and $H^3$-thymidine show that the observed RNA synthesis is associated with the follicular cells of the thyroid slice and that DNA labeling is a rare event. RNA synthesis therefore is a property of cells known to be involved in thyroglobulin synthesis and is not limited to a small population of undifferentiated cells undergoing cell division. It appeared therefore that, in this highly differentiated organ composed primarily of nondividing cells, a rapidly labeled RNA fraction is formed which with time contributes to the labeling of microsomal RNA. In the presence of actinomycin (10 $\gamma$/ml) all labeling of RNA is abolished except for a small amount associated with the 4S fraction. As seen in Fig. 2*A*, the addition of actinomycin leads to rapid inhibition of uridine incorporation into the total RNA, and this is, in fact, associated with a significant loss of label from previously synthesized RNA. Upon prolonged preincubation with 10 $\gamma$/ml of actinomycin, RNA synthesis becomes barely detectable. Two hr of preincubation with 10 $\gamma$/ml of actinomycin results in less than 1% of the RNA synthesis found in the control.

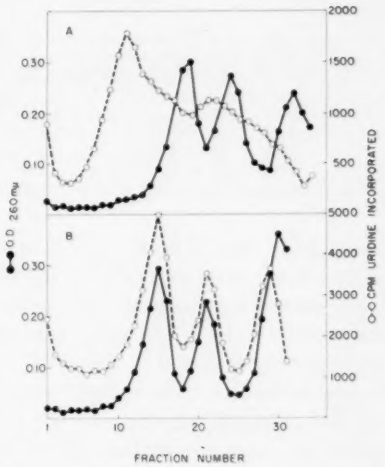


FIG. 1.—Gradient centrifugation of thyroid RNA labeled with $H^3$-uridine. Slices were incubated (*A*) 15 min in 2 ml of medium with 100 $\mu$c of $H^3$-uridine, and (*B*) 8 hr in 4 ml of medium with 200 $\mu$c of $H^3$-uridine and 2.0 $\mu$moles of unlabeled uridine. RNA was prepared for sedimentation analysis as in *Experimental*. (*A*) was centrifuged $2^3/_4$ hr and (*B*) 3 hr.

*Effect of actinomycin on the synthesis of thyroglobulin and other proteins:* Despite the abrupt shutoff in RNA synthesis due to actinomycin, the incorporation of

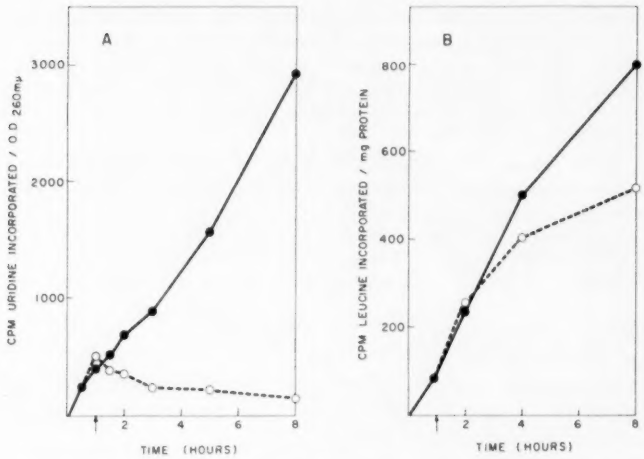


FIG. 2.—Effect of actinomycin on RNA and protein synthesis in thyroid slices. (*A*) Slices were incubated in 4 ml of medium with 40 $\mu$c of $H^3$-uridine. At the indicated times the slices were removed, blotted, and homogenized in cold 5% TCA. RNA was prepared for analysis as per Reich *et al.*[1] An aliquot was counted in Bray's solution as described. (*B*) Slices were incubated in 4 ml of medium containing 2.0 $\mu$moles of uridine and 4 $\mu$c of $C^{14}$-L-leucine. Incubation was stopped as in (*A*). Protein was worked up as per Wool and Krahl.[14] ●—● represents the control, ○- -○ represents incubations in which actinomycin (10 $\gamma$/ml) was added at 1 hr.

leucine into total cell protein continues at the control rate for about one hr and at a reduced but measurable rate for the subsequent 6 hr (Fig. 2*B*). In other experiments leucine incorporation into protein continued at almost the 6-hr rate for at least another 15 hr. The data suggested that the synthesis of some protein fraction was relatively sensitive to the action of actinomycin and linked to that of RNA, whereas some other was independent of the formation of new RNA. Support for this was obtained by studying the effect of actinomycin on the incoporation of leucine into separate fractions of the total cellular protein. A saline extract of incubated thyroid slices was fractionated by centrifugation at 27,000 $\times$ *g* into a nonextractable particulate fraction and a soluble fraction; the latter was divided by ammonium sulfate precipitation into 0–35, 35–45, and >45 per cent fractions. Whereas the incorporation of leucine into the proteins of the particulate fraction and the 0–35% ammonium sulfate fraction were sensitive to actinomycin, incorporation into the 35–45% ammonium sulfate fraction which contains the thyroglobulin was relatively resistant to actinomycin. This suggested that the synthesis of the thyroglobulin *per se* would possess considerable resistance to the action of actinomycin.

Following repeated ammonium sulfate precipitations (0–50 or 0–70 per cent) of the saline extract of incubated thyroid slices to remove nonprotein label, the 19S thyroglobulin was separated from other proteins by means of the sucrose gradient centrifugation technique. After incubation of slices with labeled leucine or $I^{125}$ (Fig. 3), the optical density peak at 280 m$\mu$ corresponding to the 19S position was labeled with the isotope. The labeled thyroglobulin was precipitable with

anti-sheep thyroglobulin antibody and, as expected from its molecular size, appeared early in the eluate from a Sephadex G200 column. The higher specific activity of $I^{125}$ on the lighter side of the 19S material (Fig. 3) was also seen with radioactive leucine and may be related to the heterogeneity of thyroglobulin.[21, 22] Whereas most of the $I^{125}$ is found to be associated with the 19S protein peak, when leucine is used as the label a large proportion of the radioactivity is associated with the 3–8S region. This is especially true when the 50–70 per cent ammonium sulfate fraction is included on the sucrose gradient centrifugation. Ammonium sulfate fractionation of the saline extract showed the 3–8S radioactivity to be comprised of material precipitating both below 35 and above 45 per cent ammonium sulfate (the latter may be related to the thyralbumin fraction described by Shulman *et al.*).[23] The incorporation of radioactive leucine into the proteins of the 3–8S fraction was considerably more sensitive to the action of actinomycin than that into thyroglobulin.

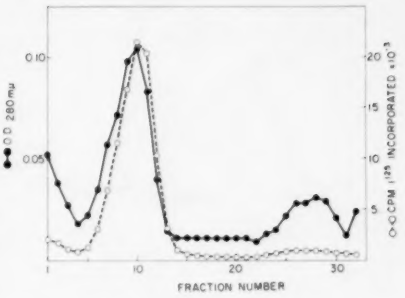


FIG. 3.—Labeling of thyroglobulin with $I^{125}$ by thyroid slices. Slices were incubated 200 min in 4 ml of medium with 0.4 μmole of L-leucine and 10 μc of $I^{125}$. Soluble protein precipitated by 70% ammonium sulfate was prepared for sedimentation analysis as in *Experimental*.

Even after preincubation with high levels of actinomycin for 5, 10, 15, and 21 hr, the labeling of thyroglobulin over the subsequent 5 hr (followed by sucrose gradient centrifugation) was not significantly different from the control which was preincubated the same length of time without actinomycin. After the first 5 hr of incubation, the rate of leucine incorporation into 19S material remains relatively constant for the next 20 hr or so. The result of a 21-hr preincubation experiment is shown in Fig. 4 (see also Figs. 6*C* and 6*D*). It can be seen, furthermore, that a 3–8S protein fraction of high specific activity is present in the 35–45 per cent ammonium sulfate fraction. This fraction whose synthesis is inhibited to a large extent by preincubation with actinomycin may be removed to a considerable degree from thyroglobulin by closer fractionation with ammonium sulfate. This probably represents contamination by proteins appearing predominantly in the 0–35 or 45–70 per cent ammonium sulfate frac-

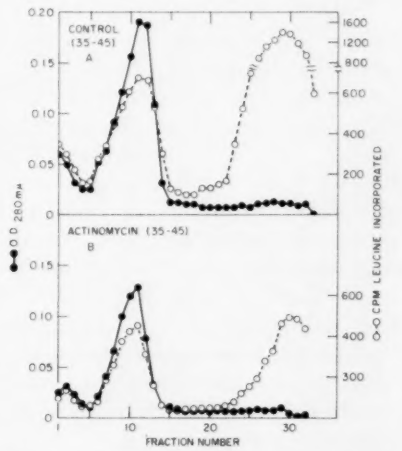


FIG. 4.—Resistance of thyroglobulin labeling to preincubation with actinomycin. Thyroid slices were incubated for 21 hr in 4 ml of medium containing 0.4 μmole of L-leucine (*A*) without and (*B*) with 10 γ/ml actinomycin. $H^3$-L-leucine (100 μc) was then added and incubation continued for 5 hr. A 35–45% ammonium sulfate fraction was prepared for sedimentation analysis as in *Experimental*.

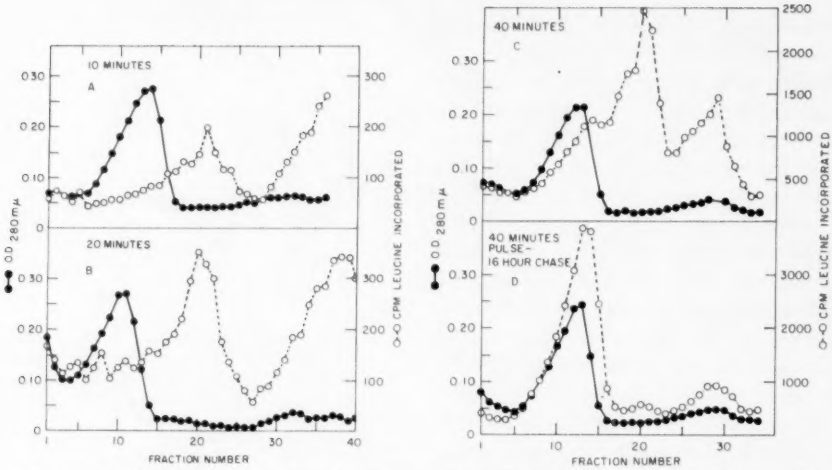


FIG. 5.—Gradient centrifugation of thyroid protein pulse-labeled with $H^3$-leucine. Slices were incubated in 4 ml of medium containing 200 $\mu$c of $H^3$-leucine and removed at (*A*) 10, (*B*) 20, (*C*) 40, and (*D*) 40 min. In (*D*) the slices were then blotted, rinsed in cold phosphate-buffered saline, and transferred to 4 ml of fresh medium containing 4 $\mu$moles/ml of unlabeled leucine. Incubation was continued for an additional 16 hr. In (*A–C*) slice protein was used, whereas in (*D*) the entire flask content was used. The soluble protein precipitated by 50% ammonium sulfate was prepared for sedimentation analysis as in *Experimental*.

tions, both of these fractions being sensitive to actinomycin. In contrast with actinomycin, puromycin rapidly inhibits labeling of thyroglobulin with leucine.

*Evidence for a thyroglobulin precursor protein:* Whereas most of the radioactive leucine was associated with 19S or 3–8S material on prolonged incubations, short periods of exposure to labeled amino acid showed a discrete peak of radioactivity in the 12S region as well as radioactivity in the lighter material (Figs. 5*A*, 5*B*, and 5*C*). With time (Figs. 5*B* and 5*C*), radioactivity appeared to move into the 19S region. Figure 5*D* shows that following a 40-min pulse, a 16-hr chase with unlabeled leucine resulted in the radioactivity becoming associated mainly with the 19S region. The label which was in the 3–8S area has changed little, whereas that in the 12S area has decreased considerably. These experiments are consistent with the thesis that the 12S substance is converted into 19S material. It should be noted that very short pulses with $I^{125}$ do lead to a slight peak of label in the 12S region, but, even at 2 min, $I^{125}$ is associated to a greater extent with thyroglobulin; in addition, there is some labeling of the lighter region. Longer incubations in the presence of radioactive leucine (Figs. 6*A*, 6*B*, and 6*C*) show a progression of radioactivity into the 19S thyroglobulin after that in the 12S fraction has reached a plateau.

Preliminary experiments with puromycin suggest that the 12S material can be converted into 19S in the absence of further protein synthesis. This is compatible with the 12S material's being a subunit precursor of thyroglobulin which does not require additional soluble RNA-ribosome interaction for conversion into thyroglobulin. By analogy with the thyroglobulin dissociation studies of Edelhoch,[25] the radioactive 12S unit formed by the thyroid slices may be half the size of the thyroglobulin molecule.

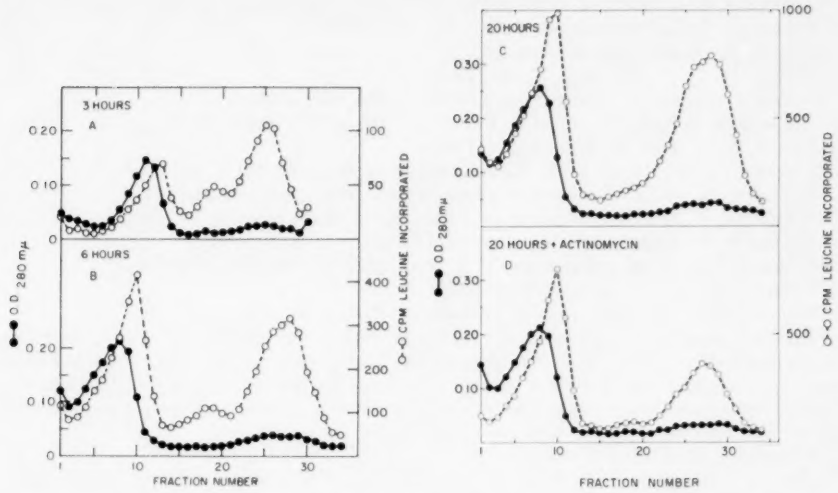


FIG. 6.—Time course of labeling of thyroid protein with $C^{14}$-leucine. Thyroid slices were incubated in 4 ml of medium with 0.4 μmole of L-leucine and 1.0 μc of $C^{14}$-L-leucine. In (*D*) 10 γ/ml of actinomycin were added at 1 hr. Protein preparation and sedimentation analysis were as in Fig. 5. The apparent small decrease in thyroglobulin specific activity due to actinomycin is actually caused by the elevated radioactivity baseline in the control incubation. This radioactivity which is inhibited by actinomycin precipitates mainly below 35% ammonium sulfate and thus was removed by the fractionation used in Fig. 4 prior to layering on the sucrose gradient.

The experiments presented here dealt primarily with the 0–50% ammonium sulfate fraction of thyroid slice extract. The 50–70 per cent fraction (3–8S material) is even more highly labeled and may contain precursor material for the 12S protein. These fractions are heterogeneous and probably contain both actinomycin-sensitive and -insensitive proteins.

*Summary*—Thyroglobulin formation by thyroid slices has been shown with the use of actinomycin to be independent of RNA synthesis. By the sucrose gradient centrifugation technique and pulse labeling with radioactive leucine, evidence was found for a 12S precursor protein in thyroglobulin synthesis.

* Supported by grants from the National Institutes of Health, and the National Science Foundation. R. W. S. was recipient of a U.S. Public Health Service postdoctoral fellowship, and I. H. G., of a Faculty Research Associate Award of the American Cancer Society.

† The following abbreviations are used: TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazoyl)-benzene; Pi, inorganic orthophosphate; TBS, Tris buffered saline containing 0.9% NaCl and 0.01 *M* Tris, pH 7.1.

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## *THE STRUCTURE OF CARBOXYPEPTIDASE A, I. A TWO-DIMENSIONAL SUPERPOSITION FUNCTION**

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*Communicated May 27, 1963*

Since bovine pancreatic carboxypeptidase A is a well-characterized enzyme of relatively low molecular weight (34,300),[1] is readily crystallized, and complexes an exchangeable metal ion at its active site,[2] it seems eminently suitable for structure analysis by the techniques of X-ray crystallography. A preliminary examination of crystals prepared by the procedure of Allan[3] was made by Kraut,[4] who concluded that the space group is $P2_1$ with one molecule per asymmetric unit. He reported cell dimensions of the zinc-containing enzyme as $a = 51.7$ Å, $b = 60.1$ Å, $c = 47.1$ Å, and $\beta = 97° 25'$, which we confirm.

*Experimental.*—Suspensions of similarly prepared carboxypeptidase A crystals (CPD-Zn), kindly supplied to us by Professor H. Neurath, were used in attempts to prepare isomorphous derivatives. Aliquots of the crystal suspensions were allowed to dialyze against LiCl-barbital buffer containing lead citrate. After several weeks, photographs showed intensity changes relative to the parent crystals. The cell dimensions of the derivative were found to be: $a = 51.5$ Å, $b = 59.9$ Å, $c = 47.2$ Å, $\beta = 97° 30'$. These values suggest that the lead derivative is isomorphous with the parent enzyme.

*Measurement of the lead derivative:* Precession photographs were taken of the (*h*0*l*) zones of the zinc enzyme and the lead derivative, and the intensities were measured photometrically on a Joyce-Loebl microdensitometer. After the standard Lorentz and polarization corrections were applied, the relative scale factor was found, and a difference Patterson map, with coefficients $(|F_{Pb}| - |F_{Zn}|)^2$, was computed (Fig. 1).

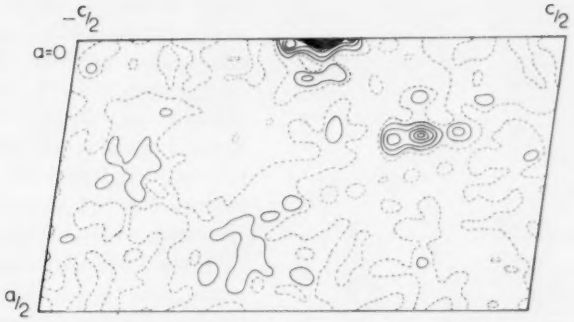


FIG. 1.—The [010] difference Patterson projection, showing peaks corresponding to two lead atoms per asymmetric unit. Broken contours are at zero, contour intervals are arbitrary.

This map indicates that the lead derivative contains two lead atoms per molecule. If $x_1$, $z_1$ and $x_2$, $z_2$ are the coordinates of the lead atoms, there should be single weight peaks in this map at $2x_1,2z_1$, and $2x_2,2z_2$, and there should be double weight peaks at $x_1+x_2$, $z_1+z_2$, and $x_1-x_2$, $z_1-z_2$. If there were two lead atoms per protein molecule, the origin peak would be expected to have a height four times that of a single weight peak due to a Pb-Pb interaction. These conditions are well satisfied by the major peaks in Figure 1.

*Difference Fourier calculations:* The atomic positions obtained from the difference Patterson map were used to calculate the signs for the coefficients to be used in difference Fourier projections. Coefficients were calculated for four atomic arrangements. In the first two calculations we assumed one lead atom at one, and then one lead atom at the other of the two proposed sites. In the third calculation we assumed both sites were occupied by a lead atom. In all of these computations full occupancy was assumed, and no temperature factor was applied. The reliability factors (all F's and f's taken as positive in this expression)

$$\frac{\Sigma\left|\left|F_{o,\,Pb}-F_{o,\,Zn}\right|-f_{Pb}\right|}{\Sigma\left|F_{o,\,Pb}-F_{o,\,Zn}\right|}$$

were 0.66, 0.62, and 0.49 in the three cases, respectively.

As a further test of the correctness of the interpretation of the difference Patterson map, another structure factor calculation was carried out, assuming the presence of a single lead atom at the site corresponding to the highest peak not at the origin in the Patterson projection (i.e., the peak previously assigned to the vectors $x_1+x_2$, $z_1+z_2$). This calculation gave a value of R = 0.54.

Four difference Fourier maps were then computed with the use of coefficients ($|F_{o,\,Pb}|-|F_{o,\,Zn}|$), modified by the sign of $f_{Pb}$. The relevant part of each map is shown in Figure 2, where the broken contour is at zero and the spacing of solid contours is arbitrary. Figures 2*a* and 2*b* both show one main peak at the position assumed and one minor peak at the position which was omitted. Figure 2*d* shows two satellite peaks separated from the main peak by a distance of 3 Å on either side of it. This is in fact the separation between the peaks in Figure 2*c* which shows no subsidiary maxima. Thus, in Figure 2*d* one atom was assumed to be present at a

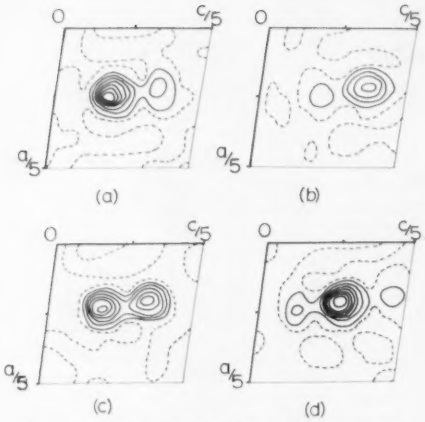


FIG. 2.—Regions of [010] difference Fourier maps: (*a*) one atom included at x = 0.094, z = 0.088; (*b*) one atom included at x = 0.086, z = 0.151; (*c*) both above atoms included; (*d*) one atom included at x = 0.090, z = 0.118.

point midway between the two correct positions. The symmetrical satellites adjacent to the assumed peak in Figure 2*d* occur because the signs are only periodically correct in reciprocal space in this example. The positions in Figure 2*c* are therefore indicated as the correct positions of the two lead sites in the molecule.

On the difference Fourier maps both peaks come up to about the same height. Had zinc been fully substituted by lead at the active site, we would expect to find in these maps an atom with the form factor, $f_{Pb} - f_{Zn}$. If one peak corresponds to full occupancy at the active site, the other corresponds to roughly 50% occupancy.

*A projection of the structure of carboxypeptidase A:* Buerger[5] proposed the use of a "ΔI" synthesis for an isomorphous pair of crystals. This synthesis should provide the superimposed images of the structure as seen from the positions of the replaceable atoms. Kartha and Ramachandran[6] pointed out that from a knowledge of the positions of these replaceable atoms, the "ΔI" synthesis could be deconvoluted to provide the crystal structure by using the Buerger minimum function. Rossmann[7] has investigated the application of this method to protein structures. He offers arguments in favor of using the sum function rather than the minimum function for deconvoluting the "ΔI" Patterson map of a protein at low resolution.

In the present case there are two sites in the asymmetric unit; it is thus possible to carry out a fourfold superposition on the "ΔI" synthesis. This procedure is sufficient to provide an image of the structure, unimpaired by pseudosymmetry, even in three dimensions.

In this paper we present a two-dimensional image of the structure projected along the *b* axis. The "ΔI" synthesis was calculated using the coefficients $(F_{Pb}^2 - F_{Zn}^2)$, and contained many positive regions apart from those corresponding to the Pb-Pb vectors. Consequently, the positions of the lead atoms on which the superposition was carried out were evaluated from the $(|F_{Pb}| - |F_{Zn}|)^2$ difference Patterson map. A two-dimensional minimum function to a resolution of 2.7 Å is shown in Figure 3. We have also calculated to a limit of 4.5 Å resolution a sum function, which shows no significant differences from the minimum function at the higher resolution. The general features of these superpositions were confirmed by the calculation of a [010] Fourier projection of the protein, in which the signs of the coefficients were evaluated by comparison of the observed differences in intensities $(F_{Pb}^2 - F_{Zn}^2)$, with the structure factors calculated from the lead positions.

*Conclusion.*—Full three-dimensional data to 6 Å resolution has been collected for the zinc enzyme both photographically and on the Arndt-Phillips Linear Dif-

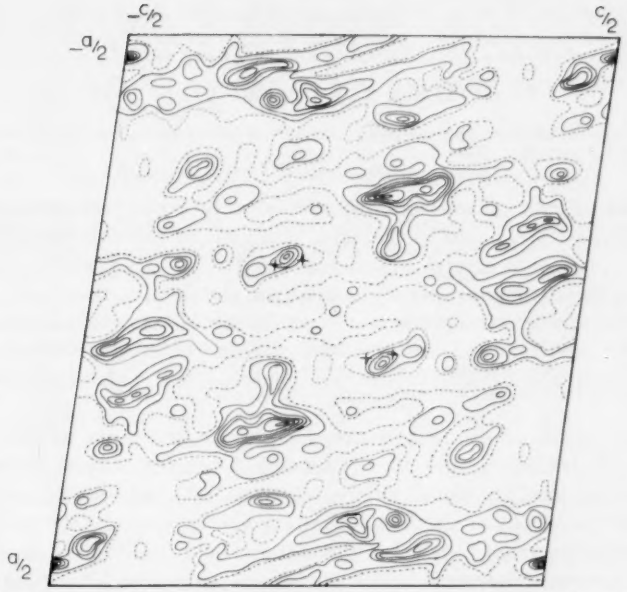


FIG. 3.—The [010] projection of the minimum function in which the origin is at the center of a full unit cell. The lead positions found from the difference Patterson map are marked by crosses.

fractometer[8] in order to extend the superposition to three dimensions. The collection of data on the lead derivative is now in progress.

The search for further derivatives continues. Dr. B. L. Vallee has recently provided us with a sample of enzyme in which the atom of zinc per molecule has been replaced by an atom of mercury. The situation therefore appears to be favorable for the application of the method of multiple isomorphous replacement to determine the phases of the protein, and this approach should eventually provide a more reliable structural model for carboxypeptidase A than the superposition method.

The authors wish to thank Professor H. Neurath, Dr. K. A. Walsh, Dr. B. L. Vallee, and Dr. J. E. Coleman for provision of material and their continuing interest in this work.

* This project has been supported by grants from the National Institutes of Health, the National Science Foundation, and the Air Force Office of Scientific Research.

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# *COMPLEMENTATION, RECOMBINATION, AND SUPPRESSION IN GALACTOSE NEGATIVE MUTANTS OF E. COLI*

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*Communicated by James F. Crow, June 3, 1963*

The metabolism of galactose in *Escherichia coli* proceeds via conversion of galactose to glucose-1-phosphate by reactions catalyzed by the enzymes galactokinase, galactose-1-phosphate uridyl transferase, and UDPgalactose-4-epimerase.[1] In addition to these reactions specific to galactose metabolism, the ability of the bacteria to utilize galactose depends on the production of catalytic amounts of UDPG and on the interconversion of glucose-1-phosphate and glucose-6-phosphate by reactions catalyzed by UDPG pyrophosphorylase and phosphoglucomutase, respectively.[1]

Mutants unable to utilize galactose for growth have been isolated by E. M. Lederberg, J. Lederberg, and M. L. Morse.[2] A number of these mutations have been classified into functional groups (cistrons) by complementation pattern in λdg partial diploids in which the temperate phage λdg provides the bacterium with "extra" galactose genes.[3] Some of the mutants have also been characterized with regard to enzymatic defects in kinase, transferase, epimerase,[4, 5] and, more recently, pyrophosphorylase activities.[6, 7] Adler and Kaiser have genetically mapped many of these mutants, utilizing recombination between transducing phage P1 and the bacterial chromosome,[8] and Morse in another mapping study has used recombination between λdg and the bacterial chromosome in λdg partial diploids.[9] Buttin has genetically mapped some independently isolated galactose negative mutants using Hfr by $F^-$ crosses.[10]

The present report describes complementation, recombination, and suppression observed in FGal partial diploids heterozygous for two different galactose negative ($Gal^-$) mutations. This study has utilized many of the "classical" Lederberg $Gal^-$ mutants plus some 60 new mutants. In addition to the genetic results, enzyme measurements for the five enzymes of galactose metabolism described above are given for some of the newly isolated mutants representative of different complementation groups.

*Materials and Methods.—Bacterial strains and crosses:* Haploid galactose negative ($Gal^-$) mutants were either representatives of the Lederberg collection (via J. Adler),[3, 8] or mutants isolated for the present study by penicillin selection and plating on EMB galactose. The new mutants were derived from a relative of the Lederberg W1 $F^-$ designated F3W1, and are designated by the prefix G (e.g., Gl, G45A, G64D). The Lederberg $Gal^-$ mutations are designated by their traditional nomenclature as $Gal_1$, $Gal_2$, etc.[3] The "W designation" of the Lederberg strains used in this work (e.g., W3102) is given in the paper of Adler and Kaiser,[8] except for $Gal_{10}$ (W4666).

Heterozygous partial diploid strains were prepared from these $Gal^-$ mutants by crosses between FGal donor bacteria (F′ strains) and $F^-$ recipients.[11, 12] The designation FGal means that the structural genes for kinase (k), transferase (t), and epimerase (e), and an operator control locus (o)— the classical Gal operon—are transferred with the F episome. The resultant partial diploid is represented as $FGal^+/Gal_b^-$ if the donor carried $FGal^+$ ($Fk^+t^+e^+o^+$) to a $Gal_b^-F^-$, or $FGal_a^-/Gal_b^-$ if the donor carried $FGal_a^-$ to a $Gal_b^-F^-$ ($Gal_a^-$ and $Gal_b^-$ refer to any mutations in the k t e o operon). The $FGal^+$ donor used was strain F′8 of Hirota.[12] Homozygous $FGal_a^-/Gal_a^-$ donor strains were prepared from heterozygous $FGal^+/Gal_a^-$ by isolation of segregant $Gal^-$ colonies. These segregant colonies, which could be either diploid $FGal_a^-/Gal_a^-$ donor strains or

haploid $Gal_a^-$, were then tested for the diploid donor state by ability to transfer an active gene to a $Gal^-$ mutant defective in a different enzyme activity. For example, presumptive kinase negative, transferase positive donors ($Fk^-t^+/k^-t^+$) were tested for ability to convert kinase positive, transferase negative $F^-$ strains ($k^+t^-$) to a galactose positive phenotype by production of $Fk^-t^+/k^+t^-$ complementing diploids.

*Complementation test:* By the above procedure homozygous FGal diploids were prepared carrying the kinase ($k^-$), transferase ($t^-$), epimerase ($e^-$), and triply defective ($o^c$-presumptive operator negative) mutations listed in Table 1. A loopful from a Penassay broth culture of the $Gal^-F^-$ strain to be tested for complementation group was spotted in a number of places on an EMB galactose plate (on which bacteria unable to utilize galactose for growth make pale colonies). Loopfuls from cultures of the $Fk^-$, $Ft^-$, $Fe^-$, or $Fo^c$ $F'$ donor strains were then spotted on top of the $F^-$ bacteria. Genetic transfer from $F'$ to $F^-$ ensued, and in every case in which complementation could occur (e.g., $Fk^-t^+/k^+t^-$) solid dark growth spots indicative of galactose fermentation appeared after overnight incubation. Where complementation could not occur (e.g., most $Fk_1^-/k_2^-$), the resultant spots were pale. After about 48 hr, recombination producing $Gal^+$ genes could be detected in noncomplementing cases by the appearance of isolated dark dots in the pale spot. This low frequency recombination phenomenon could be readily differentiated from the complementation response.

TABLE 1

COMPLEMENTATION CLASSIFICATION AND ENZYMATIC CHARACTERIZATION OF $GAL^-$ MUTANTS

| Mutation | Complementation group | Enzyme activities in recipient strains k | t | e | pp | m |
|---|---|---|---|---|---|---|
| F3W1 | Wild type | 5.9 | 11 | 22 | 2.2 | 14 |
| $Gal_{20}$, $Gal_{24}$, $Gal_{10}$,* $Gal_2$,* $Gal_3$,* $Gal_{12}$,* $Gal_{14}$* | K | — | + | + | | |
| G6A | K | <0.1 | 23 | 23 | 2.2 | 18 |
| G47C | K | <0.1 | 19 | 25 | 1.5 | 17 |
| $Gal_1$,* $Gal_4$* | T | + | − | + | | |
| G15B | T | 2.5 | <0.2 | 17 | 1.2 | 4.2 |
| G45B | T | 2.3 | 0.7 | 17 | 1.1 | 4.6 |
| $Gal_{16}$,* $Gal_{22}$* | E | + | + | − | | |
| G47A | E | 6.3 | 16 | 5.3 | 3.0 | 13 |
| $Gal_5$,* $Gal_9$* | O | − | − | − | | |
| G34 | NFI | 11 | 19 | 20 | <0.1 | 8.4 |
| G64D | NFI | 5.4 | 12 | 22 | <0.1 | 6.2 |
| GI | NFII | 3.1 | 7.3 | 5.9 | 2.3 | 12 |
| G45A | NFII | 3.0 | 12 | 6.3 | 2.9 | 17 |

Complementation classification is based upon data as in Table 2. A mutation is classified in the K complementation group if it exhibits no "true" complementation (see text) with any $k^-$ mutation and complements $t^-$ and $e^-$. A similar basis is used to define the T and E groups. A mutation is classified in the O complementation group if it exhibits no true complementation with $k^-$, $t^-$, or $e^-$, but a recipient O strain becomes phenotypically $Gal^+$ after acquisition of $Fk^+t^+e^+o^+$. A mutation is classified as NF (not F-ducible to $Gal^+$) if the recipient strain does not become phenotypically $Gal^+$ after acquisition of $Fk^+t^+e^+o^+$. Strains marked by asterisks (e.g., $Gal_{10}$*) have been studied for complementation patterns by E. M. Lederberg using λdg.[3] The symbol + or − under enzyme activity refers to previously published data of Kalckar *et al.*[4] and Soffer[5] for kinase (k), transferase (t), and epimerase (e). The designations pp and m under enzyme activities stand for UDPG pyrophosphorylase and phosphoglucomutase, respectively. Numerical values under enzyme activities refer to measurements made in the present study. Units of enzyme activity are μmoles product/hr/mg protein.

*Recombination measurements:* Recombination frequency between noncomplementing mutations was measured quantitatively. In genetic transfer experiments in liquid medium used for recombination measurements, more than 99% of the $F^-$ cells received the FGal region under the conditions employed (10/1 excess of $F'/F^-$).[13] Therefore, the frequency with which recombinant $Gal^+$ bacteria are produced in a population of noncomplementing diploids after a cross of $FGal_a^-/Gal_a^-$ by $Gal_b^-$ $F^-$ should provide a measure of the distance between the markers $Gal_a^-$ and $Gal_b^-$ in the usual genetic sense. $Gal^+$ recombinants were scored for this purpose on plates containing galactose as sole carbon source.[13] A number of crosses were performed between $k^-$, $t^-$, and $e^-$ mutants and $o^c$ mutants (there is a complementation interaction of $o^c$ with $k^-$, $t^-$, or $e^-$ only in the rare cases described below). Reciprocal crosses were used in an effort to minimize any special features of the episomal gal genes.

*Enzyme assays:* Kinase assays were performed as described by Sherman and Adler;[14] transferase and epimerase assays as described by Kalckar *et al.*,[4] except that the single step epimerase assay was done in the presence of KCN;[15] pyrophosphorylase and phosphoglucomutase assays as described by Fukasawa *et al.*[16] Mutant cultures were grown for assay in tryptone broth[8] with $10^{-2}$ *M* galactose as inducer, centrifuged in late log phase and washed, resuspended, and sonnicated in potassium phosphate-mercaptoacetic acid buffer.[14] Sonnication was carried out for 5 min in a Raytheon 10 KC oscillator. All assays were performed on at least two separate extracts.

Galactose-1,6-diphosphate was the gift of H. A. Lardy. $C^{14}$-galactose was purchased from Volk Radiochemicals. Other substrates, cofactors, and enzymes used in the assays were obtained from the Sigma Chemical Company.

*Results.—Complementation tests:* The episome designated FGal includes the structural genes for kinase, transferase, and epimerase, and a genetic site controlling formation of all three enzymes—a presumptive operator locus.[17] Homozygous FGal donor strains were prepared carrying seven known kinase ($k^-$) mutations, two transferase ($t^-$) mutations, an epimerase ($e^-$) mutation, and two triply defective ($o^c$) mutations (see *Methods*). An FGal donor strain carrying $Fk^+t^+e^+o^+$ was already available.[12] These donor strains were then used to introduce their episomal genetic material containing the structural genes for kinase, transferase, and epimerase into various $Gal^-$ $F^-$ strains, producing partial diploids. The resultant strains possess only a small amount of donor genetic material; genes unlinked to the k t e o genetic region are not cotransferred.

Recipient mutant strains could be divided into those which gave a $Gal^+$ phenotype after mating with $Fk^+t^+e^+o^+$, and those which failed to give a $Gal^+$ phenotype even though they received the $Fk^+t^+e^+o^+$ episome (as judged by subsequent transfer of $Fk^+t^+e^+o^+$). The class which was not F-ducible to $Gal^+$ presumably possessed a defect outside of the genetic region carried by the FGal episome—outside of the classical "Gal region" containing the structural genes for kinase, transferase, and epimerase.

The class of $Gal^-$ mutants which gave a $Gal^+$ phenotype after transfer of $Fk^+t^+e^+o^+$ were then further classified as to $Gal^+$ or $Gal^-$ phenotype after transfer of $Fk^-(Fk^-t^+e^+o^+)$, $Ft^-(Fk^+t^-e^+o^+)$, or $Fe^-(Fk^+t^+e^-o^+)$ from donor strains. If the resultant "trans" diploid was phenotypically $Gal^-$ with, for example, $Fk^-$, but $Gal^+$ with $Ft^-$ and $Fe^-$, the $Gal^-$ mutant being tested could be classified as belonging to the kinase complementation group with a mutation in the kinase cistron.[18]

*Complementation between mutants having previously determined single enzyme defects:* The Lederberg mutants with known single enzymatic defects were first tested for complementation. Most of the results were consistent with the previously determined missing enzyme activities. All $k^-$ mutations complemented $t^-$ and $e^-$ to give a $Gal^+$ phenotype; all $t^-$ complemented $k^-$ and $e^-$; and $e^-$ complemented $k^-$ and $t^-$. This was true regardless of whether a given $Gal_a^-$ ($k^-$, $t^-$, or $e^-$) mutation was introduced into recipient $Gal_b^-$ bacteria, or $Gal_b^-$ was introduced into recipient $Gal_a^-$ in the reciprocal experiment. In general, $k^-$ mutations did not complement with other $k^-$, nor $t^-$ with $t^-$, nor $e^-$ with $e^-$. Some typical results are given in Table 2, and the complementation classification in Table 1.

*Suppression effects:* One exception to the lack of complementation between mutations affecting a given enzyme was found. A strong $Gal^+$ response was observed in spot tests involving the $Fk^-$ donor $Gal_{20}$ and the $k^-$ recipient $Gal_{10}$. $Gal_{20}$ as a donor complemented no other $k^-$ recipient, and $Gal_{10}$ as a recipient comple-

TABLE 2

SAMPLE DATA INDICATING BASIS OF COMPLEMENTATION CLASSIFICATION

| Recipient genotype | Donor genotype introduced by FGal | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $k_2^-$ | $k_{10}^-$ | $k_{20}^-$ | $k_8^-$ | $t_1^-$ | $t_4^-$ | $e_{16}^-$ | $o^\circ_3$ | $o^\circ_9$ | $k^+t^+e^+o^+$ |
| $k_2^-$ | − | − | − | − | + | + | + | − | − | + |
| $k_{10}^-$ | − | − | + | − | + | + | + | − | − | + |
| $k_{20}^-$ | − | − | − | − | + | + | + | − | − | + |
| $k_8^-$ | − | − | − | − | + | + | + | − | − | + |
| $t_1^-$ | + | + | + | + | − | − | + | − | − | + |
| $t_4^-$ | + | + | + | + | − | − | + | − | − | + |
| $e_{15}^-$ | + | + | + | + | + | + | − | − | − | + |
| $o^\circ_2$ | − | − | + | − | − | + | wk+ | − | − | + |
| $o^\circ_9$ | − | − | − | − | − | − | − | − | − | + |
| k⁻G6A | − | − | + | − | + | + | + | − | − | + |
| t⁻G15B | + | + | + | + | − | + | + | − | − | + |
| PP⁻G34 | − | − | − | − | − | − | − | − | − | − |

Each donor was spotted with each Gal⁻ recipient on an EMB galactose plate as described under *Methods*. A resultant Gal⁺ phenotype in the ensuing diploid is designated by +. A resultant Gal⁻ phenotype is designated by −. The donor genotype $k_2^-$ abbreviates $k_2^-t^+e^+o^+$, $t_1^-$ abbreviates $k^+t_1^-e^+o^+$, etc. The recipient genotype $k_2^-$ abbreviates $k_2^-t^+e^+o^+pp^+$, etc., where $k^-$, $t^-$, $e^-$, and $pp^-$ refer to loss of kinase, transferase, epimerase, or pyrophosphorylase activity, respectively. The pyrophosphorylase gene is not carried by the FGal episome.

mented no other k⁻ donor. This result indicated either complementation within the kinase cistron or a suppression of one of the k⁻ mutations. In the reciprocal complementation test with the $Gal_{10}$ mutation introduced from a donor strain and the $Gal_{20}$ mutation in a recipient, no complementation was observed (see Table 2). This showed that the genetic material involved in producing the Gal⁺ phenotype in the $FGal_{20}/Gal_{10}$ partial diploid was not included in the $FGal_{10}/Gal_{20}$ partial diploid. The $Gal_{10}F^-$ recipient strain then apparently possesses a suppressor locus unlinked to the kinase gene which can act to reverse the effect of the $Gal_{20}$ defect. The suppressor gene is not incorporated by the FGal episome carrying $Gal_{10}$ and therefore not transferred in the formation of the $FGal_{10}/Gal_{20}$ partial diploid. The test for reciprocal complementation then serves to distinguish between "true" complementation between galactose defects and effects due to an unlinked suppressor gene.

*Complementation tests with presumptive o° mutants:* No complementation between any of the k⁻, t⁻, or e⁻ mutations and the triply defective mutation $Gal_9$ was observed. This result is consistent with the concept of this mutation as an operator negative (o°) control defect turning off the k, t, and e structural genes. In complementation experiments with the triply defective mutant $Gal_3$ as a recipient, a strong Gal⁺ response was observed in spot tests with the Fk⁻ donor $Gal_{20}$ and the Ft⁻ donor $Gal_4$, and a weak Gal⁺ response was obtained with the Fe⁻ donor $Gal_{16}$. However, in reciprocal experiments in which the $Gal_3$ mutation was introduced into recipient $Gal_{20}$, $Gal_4$, and $Gal_{16}$, no complementation was observed. This lack of reciprocal complementation indicates the presence of a suppressor mutation in the $Gal_3$ F⁻ strain which is active on the $Gal_{20}$, $Gal_4$, and $Gal_{16}$ defects. The absence of any "true" complementation between $Gal_3$ and k⁻, t⁻, and e⁻ suggests that this mutation is an operator negative control defect like the $Gal_9$ mutation.

*Complementation and suppression in newly isolated mutants:* Sixty independently isolated mutants from the F3W1 Gal⁺ strain were tested, first for conversion to Gal⁺ by acquisition of $Fk^+t^+e^+o^+$ and then for complementation with the Gal⁻ donors having known defects. More than half of these mutants (33) did not acquire a standard Gal⁺ phenotype (as judged by colony color on EMB galactose

plates) upon receipt of the $k^+t^+e^+o^+$ genetic region from the $FGal^+$ donor. These strains which were not $F^-$ducible to $Gal^+$ could be divided into two distinct groups on the basis of growth on EMB galactose plates and response to F-duction by $Fk^+t^+e^+o^+$. One group (designated NFI) grows very poorly on EMB galactose plates as a haploid and exhibits markedly poorer growth after acquisition of $Fk^+t^+e^+o^+$. There appears to be a galactose sensitivity, enhanced by the "extra" galactose genes brought in by the episome. The other group (designated NFII) are all "leaky" mutants exhibiting some growth on plates containing galactose as sole carbon source. This group of mutants grows very well on EMB galactose plates. After acquisition of the $Fk^+t^+e^+o^+$ the colonies are darker but distinctly not wild type, so that the NFII group (in contrast to NFI) is more nearly normal with the "extra" galactose genes. The enzymatic characterization of these mutants is discussed below.

The 27 mutant strains F-ducible to $Gal^+$ by $Fk^+t^+e^+o^+$ were further classified by complementation in spot tests with $Fk^-$, $Ft^-$, $Fe^-$, and $Fo^\circ$ donors. Eleven of the mutants were defective in the kinase (K) cistron, 15 in the transferase (T) cistron, and one "leaky" mutant (G47A) was tentatively classified as partially defective in the epimerase (E) cistron and containing a suppressor gene able to reverse the effect of the $Gal_{16}$ defect. All mutants defective in the kinase cistron were able to suppress the kinase defect $Gal_{20}$, and all mutants defective in the transferase cistron were able to suppress the transferase defect $Gal_4$, as judged by reciprocal complementation experiments. Therefore, the F3W1 strain apparently possesses a "natural" suppressor locus unlinked to the structural genes for kinase, transferase, and epimerase which can reverse the effect of the $Gal_{20}$, $Gal_4$, and $Gal_{16}$ mutations.

*Recombination experiments:* The recombination distance between a number of the Lederberg kinase, transferase, and epimerase mutations and the operator negative mutations was determined (see *Methods*). The frequencies of $Gal^+$ recombination between the $o^\circ$ $Gal_9$ and mutations representative of the kinase, transferase, and epimerase cistrons are given in Figure 1. Recombination distances measured from the $o^\circ$ $Gal_3$ give essentially the same map. Kinase mutations are "far" from the o site; epimerase mutations are "close"; and transferase mutations tend to fall at an intermediate genetic recombination distance. It should be noted that the *order* of mutations is arbitrary—only the *distance* from $o^\circ$ is measured in these recombination experiments. The experiments do not determine on which "side" of $o^\circ$ a given mutation lies.

*Enzyme activity measurements:* Nine of the galactose negative mutants isolated from the F3W1 $Gal^+$ strain were investigated for possible enzyme defects in kinase, transferase, epimerase, pyrophosphorylase, and phosphoglucomutase. The mutants studied enzymatically were selected from those previously classified by complementation tests as typical of a particular complementation group: K, T, E, NFI, or NFII. The results of the enzyme assays are given in Table 1.

The mutants G6A and G47C classified as defective in the K cistron by complementation tests are clearly very low in kinase activity; G15B and G45B of the T cistron are low in transferase; and G47A of the E cistron is low but not absent in epimerase activity (as would be expected from its "leaky" nature). The NFI (not F-ducible to $Gal^+$) mutants G34 and G64D are defective in UDPG pyrophosphoryl-

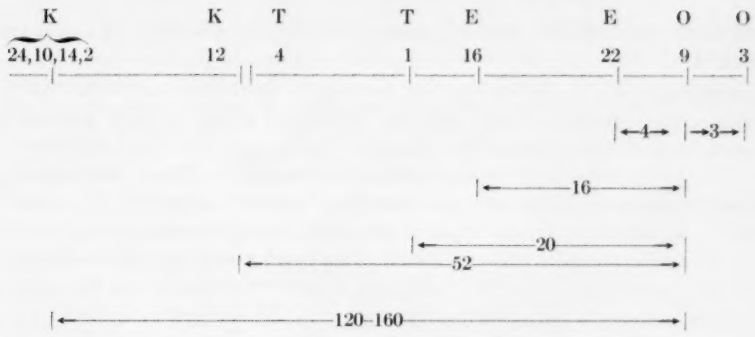


FIG. 1.—Recombination frequency of kinase, transferase, and epimerase mutations with operator negative mutations. Crosses were performed as described under *Methods*. The gene order is arbitrary. The numbers in the figure represent 100 times the observed per cent of recombination in the partial diploid recipient population.

ase (pp) activity. A similar defect in Leberberg strains not λdg transducible to Gal$^+$ has recently been described.[6, 7] The galactose sensitivity of these mutants probably results from the accumulation of galactose-1-phosphate because no UDPG is available for the transferase reaction. In this connection it should be noted that those strains which accumulate galactose-1-phosphate, the transferase and pyrophosphorylase defectives, exhibit reduced activity of phosphoglucomutase (m). This supports the concept that the physiological basis of galactose toxicity in these "galactosemic" bacteria may be the inhibition of phosphoglucomutase and other enzymes which catalyze reactions involving glucose-6-phosphate[19, 20] or glucose-1-phosphate.

The NFII (not F-ducible to Gal$^+$) mutants G1 and G45A are low in epimerase activity. However, these mutants should be F-ducible to Gal$^+$ by Fe$^+$ strains, if the low epimerase activity indicates a defect in the known epimerase structural gene. The NFII strains could be double mutants, with a leaky mutation in the epimerase cistron and a second mutation outside of the k t e o operon. Since kinase, transferase, pyrophosphorylase, and mutase activities (and galactose uptake) are all near normal levels, it is difficult to imagine what other defect of galactose metabolism these mutants could have. An alternative explanation is that there are two cistrons controlling epimerase activity, with either two separate enzymes or two polypeptide chains and a single enzyme. The second cistron (presumably defective in these mutants) would have to be outside of the genetic region carried by the FGal episome. Analysis of the defect in the NFII mutants is hampered because of the considerable "leakiness" of these strains with regard to growth with galactose as energy source and with regard to epimerase activity.

The level of kinase activity was also measured in extracts from two partial diploid strains prepared by addition of $FGal_{20}$ to the recipient strains $Gal_{10}$ and G6A which have suppressors able to reverse the $Gal_{20}$ defect. The partial diploids were checked in each case for the $FGal_{20}/k^-$ property by $FGal_{20}$ transfer complementation pattern and $Gal_{10}$ or G6A recipient complementation pattern in Gal$^-$ segregants. The kinase activity in the suppressed $FGal_{20}/k^-$ strains was as high as in the control $Fk^+/k^-$ strain, so that the structural gene totally inactive in producing functional en-

zyme in the original Gal⁻ strain acquires full activity as a result of the suppressor action.

*Discussion.*—E. M. Lederberg has previously described complementation classification for a number of Gal⁻ mutants by the use of λdg to make partial diploids (strains marked with an asterisk in Table 1).[3] We obtain an identical classification with FGal complementation tests except that we find no "true" complementation between epimerase (E group) mutations and operator negative (O group) triply defective mutations. In the original λdg work, complementation between e⁻ and triply defective mutants was reported.[3] However, recent unpublished λdg studies by E. M. Lederberg (quoted by Adler and Kaiser[8]) have indicated noncomplementation, in agreement with our findings and with the concept of these mutations as operator negative control defects inactivating the k t e o operon.[17] The suppressor sensitive mutation $Gal_{20}$ was listed as "unclassified" on the basis of λdg complementation tests.[3]

There is complete accord between complementation classification and enzyme defect in all mutants amenable to complementation analysis, indicating that the FGal complementation test provides a simple and accurate determination of enzyme defect. We have found no evidence for the intracistron complementation described in Neurospora[21, 22] and other bacterial genetic systems,[23, 24] although a thorough search has been made only in the case of the kinase mutations.

Our recombination study agrees in general with the mapping experiments of Adler and Kaiser[8] and Buttin[10] in that kinase mutations are "far" from the operator site, epimerase mutations are "close," and transferase mutations are an intermediate recombination distance from o. The recombination data reported in this paper do not allow a determination of gene order. Morse has performed a mapping study similar to ours, using recombination between k⁻ or t⁻ mutations and o° in λdg diploids.[9] He finds k⁻ mutations are slightly further from o° than t⁻ on the average, but obtains overlap in distance between k⁻ and t⁻.

The suppressor locus active on galactose mutations described here may be the same as the suppressor active on alkaline phosphatase mutations described by Garen and Siddiqi in the W1 strain.[25] The high level of kinase activity found in the suppressed mutants is similar to the high level of enzyme activity in some suppressed alkaline phosphatase mutants. Current theories of suppression implicate altered transfer RNA specificity as responsible for restored enzymatic activity in suppressed mutants.[26, 27] The "natural" suppressor in the W1 strain may be the gene for an "extra" transfer RNA molecule for a particular amino acid, so that a nonsense alteration in the donor Gal⁻ strain becomes a functional code unit in the W1 recipient.

*Summary.*—Combined genetic complementation and enzymatic activity data show that five functionally distinct groups of galactose negative mutations occur in *E. coli*, corresponding to loss of kinase, transferase, epimerase, or pyrophosphorylase activity, or to a loss of kinase, transferase, *and* epimerase activity via operator negative type defects. Recombination experiments indicate that the epimerase defects are close to the operator site, transferase defects are an intermediate genetic distance, and kinase defects are farthest away from the operator. A suppressor locus is described which leads to restored function of certain Gal⁻ mutations.

We wish to thank Karen Rutherford, Erna Rollefson, Charles Hill, and Douglas MacPherson for assistance in various phases of these experiments, and Julius Adler for the Lederberg mutants and helpful comments.

The following abbreviations are used: Gal, galactose; Gal-1-P, α-D-galactose-1-phosphate; G-1-P, α-D-glucose-1-phosphate; G-6-P, glucose-6-phosphate; UDPGal, uridine diphosphogalactose; UDPG, uridine diphosphoglucose; ATP and ADP, adenosine triphosphate and adenosine diphosphate; UTP, uridine triphosphate; PP, pyrophosphate.

* This work was supported by grant GM 08407 from the National Institutes of Health (U.S. Public Health Service).

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## *A GENERALIZATION OF BOUNDED HAAR MEASURE*

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*Communicated June 28, 1963*

We are going to introduce a further generalization, a simplifying one, into our previous[1] generalization of a theorem of Helson-Lowdenslager. A somewhat specialized case of an alternate version of the theorem of the present paper has been stated in another context without proof.[2]

We introduce, and hold fast a set of objects

$$T, \Sigma, P, \Phi, \Psi$$

of the following description.

(*i*) $T = \{t\}$ is a general pointset, and $\Sigma = \{X\}$, $X \subset T$, is some sigma-algebra of subsets of $T$.

(*ii*) $P = \{\nu\}$ is the family of (finite) nonnegative sigma-additive set functions $\nu(X)$ on $(T, \Sigma)$, $\nu(X) \geq 0$.

(*iii*) $\Phi = \{\varphi\}$ is some complex-linear ring of bounded measurable functions on $(T, \Sigma)$, so that, in symbols

$$c\,\Phi \subset \Phi, \qquad \Phi + \Phi \subset \Phi, \qquad \Phi \cdot \Phi \subset \Phi.$$

(*iv*) $\Psi = \{\psi\}$ is the *largest* family of bounded measurable functions for which

$$\Psi \cdot \Phi \subset \Phi; \tag{1}$$

that is, if $\varphi \in \Phi$, $\psi \in \Psi$, then $\psi \cdot \varphi \in \Phi$.

*Remark 1:* $\Psi$ is likewise a complex-linear ring. It includes the elements of $\Phi$ and all constant functions on $T$, and it now takes the place of the "composite" ring $\Phi + \Gamma$ of the previous paper.[1]

We again denote by $\Phi^* = \{\varphi^*\}$ the ring with elements conjugate complex to those of $\Phi$. For any $\nu$ in $P$, $L_2(\nu)$ is the customary $L_2$-space on $(T, \Sigma, \nu)$, and $\Phi_2(\nu)$, $\Psi_2(\nu)$, etc., are the subspaces of $L_2(\nu)$ which are spanned by $\Phi$, $\Psi$, etc.

*Definition 1:* An element $\sigma$ of $P$ is said to be orthogonal to $\Phi^*$ if

$$\int \varphi d\sigma = 0, \quad \varphi \in \Phi. \tag{2}$$

*Remark 2:* Since $\sigma$ is real-valued, if it is orthogonal to $\Phi^*$, it is also orthogonal to $\Phi$, that is,

$$\int \varphi^* d\sigma = 0, \; \varphi \in \Phi.$$

*Remark 3:* Relation (1) implies that if $\sigma$ is orthogonal to $\Phi^*$, then the subspaces $\Phi_2(\sigma)$, $\Psi_2^*(\sigma)$ of $L_2(\sigma)$ are perpendicular to each other.

*Definition 2:* We say that $\sigma$ is a *basic measure* if (*i*) it is orthogonal to $\Phi$ and (*ii*) it is "maximal" in the sense that

$$\Phi_2(\sigma) \otimes \Psi^*(\sigma) = L_2(\sigma).$$

*Remark 4:* In Definition 2 we have not excluded the case $\sigma(T) = 0$.

*Definition 3:* We say that $\sigma$ is a *generalized Haar measure* if (*i*) it is a basic measure, (*ii*) $\sigma(T) > 0$, and (*iii*) any element $\nu$ of $P$ which is orthogonal to $\Phi$ is a constant multiple of $\sigma$.

We again[1] associate with any $\nu \in P$ the number

$$M(\nu) = \inf \int |1 - \varphi|^2 d\nu, \; \varphi \in \Phi, \tag{3}$$

$0 \leq M(\nu) < \infty$. There exists[1] a Baire function $F_0 = F_0(t)$ which is determined except on a set of $\nu$-measure 0 for which

$$M(\nu) = \int |1 - F_0|^2 d\nu, \tag{4}$$

and it is characterized by

$$\int (1 - F_0^*)\varphi d\nu, \; \varphi \in \Phi.$$

*Remark 5:*[3] If $\nu \geq \nu'$ $(\geq 0)$, then $M(\nu) \geq M(\nu')$. More generally, if $\nu \geq \nu' + \nu''$, then $M(\nu) \geq M(\nu') + M(\nu'')$.

After fixing $F_0$ in (4) uniquely, we again define $X_a$ and $X_s$ by

$$|1 - F_0|^2 > 0 \text{ for } t \in X_a, \ |1 - F_0|^2 = 0 \text{ for } t \in X_s,$$

$$X_a \cup X_s = T, \ X_a \cap X_s = 0,$$

and we again introduce the decomposition

$$\nu = \nu_a + \nu_s$$

where

$$\nu_a(X) = \nu(X \cap X_a), \ \nu_s(X) = \nu(X \cap X_s).$$

*Remark 6:*[4] We have $M(\nu_a) = M(\nu)$, $M(\nu_s) = 0$. If $M(\nu') = M(\nu)$ for $0 \leq \nu' \leq \nu$, then $\nu_a \leq \nu' \leq \nu$.

*Definition 4:* For each $\nu \in P$ we again denote by $\sigma_\nu$ the nonnegative measure

$$\sigma_\nu(X) = \int_X |1 - F_0|^2 d\nu, \ X \subset \Sigma,$$

or in "differential" form

$$d\sigma_\nu = |1 - F_0|^2 d\nu = |1 - F_0|^2 d\nu_a.$$

*Remark 7:*[5] For each $\nu$, the associated measure $\sigma_\nu$ is orthogonal to $\Phi$.

We again introduce on $X_a$ the quotients $(1 - F_0)^{-1}$, $(1 - F_0^*)^{-1}$ and their product

$$(h_\nu \equiv) \ h(t) = |1 - F_0|^{-2},$$

and we again complete these functions on $X_a$ to functions on all of $T$ by giving them values 0 on $X_s$, so that we again can put

$$d\nu_a = h d\sigma_\nu. \tag{5}$$

*Remark 8:*[6] (*i*) The functions $(1 - F_0)h$ and $(1 - F_0)^{-1}$ are both in $L_2(\sigma_\nu)$. (*ii*) The function $(1 - F_0)^{-1}$ is orthogonal to $\Phi_2^*(\sigma_\nu)$. Thus, (*iii*) if it so happens that $\sigma_\nu$ is a basic measure in the sense of Definition 2, then $(1 - F_0)^{-1}$ lies in $\Psi_2(\sigma_\nu)$.

We now again take a fixed element $\nu \in P$, with its associated objects $F_0$, $X_a$, $X_s$, $\nu_a$, $\nu_s$, $\sigma_\nu$, $h$, and we again make the express assumption that $\sigma_\nu$ is a basic measure.

This being given fixed, we take any complex-valued sigma-additive set function $\mu$ on $(T, \Sigma)$ for which

$$|\mu(X)| \leq C\nu(X), \ X \subset \Sigma, \tag{6}$$

where $C$ is a finite positive constant.

*Definition 5:* We say that $\mu$ is analytic if

$$\int \varphi d\mu = 0, \ \varphi \in \Phi, \tag{7}$$

and we say that it is strictly analytic if

$$\int \Psi d\mu = 0, \ \psi \in \Psi.$$

Let $\mu$ be any complex set function with (6). If we decomposite it into the sum

$$\mu = \mu_a + \mu_s$$

where

$$\mu_a(X) = \mu(X \cap X_a),\ \mu_s = \mu(X \cap X_s), \tag{8}$$

then the following theorem holds.

THEOREM. *If $\mu$ is analytic, then $\mu_a$ is also analytic, and $\mu_s$ is even strictly analytic.*

*Proof:* Since $\Phi$ is a ring, we can replace $\varphi$ in (7) by $(1 - \varphi_0)\varphi$, and then $\varphi_0$ by $F_0$, so that

$$0 = \int (1 - F_0)\varphi d\mu,\ \varphi \in \Phi.$$

Next, by (8), we can write for this

$$0 = \int (1 - F_0)\varphi d\mu_a,$$

and finally, by (1), we even have

$$0 = \int (1 - F_0)\psi\varphi d\mu_a,$$

for all $\varphi \in \Phi$, $\psi \in \Psi$. Due to (5) and (6), we have

$$d\mu_a = \delta(t)h(t)d\sigma_\nu,\ |\delta(t)| \leq C, \tag{9}$$

and thus,

$$0 = \int [(1 - F_0)h(t)]\psi \cdot \delta(t)\varphi \cdot d\sigma_\nu.$$

Herein $\delta(t)\varphi$ is bounded, and $(1 - F_0)h \in L_2(\sigma_\nu)$ by Remark 8. Since $\psi$ is "arbitrary," we may replace it by any element of $\Psi_2(\sigma_\nu)$, and by part (*iii*) of remark 8 we may therefore replace $\psi$ by $(1 - F_0)^{-1}$. Now if we do this, we obtain

$$0 = \int h(t) \cdot \delta(t) \cdot \varphi d\sigma_\nu,$$

and thus, by (9),

$$0 = \int \varphi d\mu_a. \qquad \varphi \in \Phi.;$$

that is, $\mu_a$ is analytic.

Since $\mu$ itself was analytic to begin with, the difference $\mu_s = \mu - \mu_a$ is analytic, at any rate. Thus,

$$0 = \int \varphi d\mu_s,\ \varphi \in \Phi.$$

By (1), we can herein replace $\varphi$ by $\psi\varphi$ where $\psi \in \Psi$, and therefore

$$\int \psi d\mu_s = \int \psi(1 - \varphi)d\mu_s.$$

However,

$$\int |\psi(1 - \varphi)d\mu_s|^2 \leq \int |\psi|^2 d\nu_s \cdot \int |1 - \varphi|^2 d\nu_s,$$

and thus, if we hold $\psi$ fast and vary $\varphi$, then by (3) we obtain

$$\left|\int \psi d\mu_s\right|^2 \leq \int |\psi|^2 d\nu_s \cdot M(\nu_s).$$

But, by Remark 6, $M(\nu_s) = 0$, and therefore

$$\int \psi d\mu_s = 0 \qquad \psi \in \Psi;$$

that is, $\mu_s$ is strictly analytic, as asserted.

COROLLARY.[7] *If there exists a generalized Haar measure in the sense of Definition 3; if, on denoting it by* $\sigma_0$, *we introduce the normalization* $\sigma_0(T) = 1$; *if for any sigma-additive complex set function* $\mu$ *we introduce the decomposition*

$$d\mu = wd\sigma_0 + d\mu_s$$

*in which* $w \in L_1(\sigma_0)$ *and* $\mu_s$ *is singular relative to* $\sigma_0$; *and if* $\mu$ *is analytic; then* $\mu_s$ *is strictly analytic.*

* This research was supported by the U.S. Air Force Office of Scientific Research.

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[2] Bochner, S., "Analytic measures on compact Bohr groups," to appear.

[3] See Theorem 4 of reference 1.

[4] See Theorem 5 of reference 1.

[5] See Theorem 6 of reference 1.

[6] See Theorems 7 and 8 of reference 1.

[7] See Theorem 10 of reference 1.

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## *HOST-INDUCED MODIFICATION OF T-EVEN PHAGES DUE TO DEFECTIVE GLUCOSYLATION OF THEIR DNA**

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*Communicated by S. E. Luria, June 26, 1963*

Host-induced modifications (HIM) are changes in the host range of viruses brought about by growth in specific hosts.[1] These changes are typically reversible, being phenotypic rather than genotypic. Yet, recent work has shown that in phage λ the host-induced changes affect the phage deoxyribonucleic acid (DNA).[2] The first case of HIM described[3] was one in which phages T2 and T6 were changed to T*2 and T*6, respectively, by growth in certain bacterial strains, called $B/4_0$, which had been isolated from *Escherichia coli* B as resistant to phages T4, T3, and T7. Phages T*2 and T*6 lack the ability to multiply in *E. coli* B and in its variants, but can multiply in strains such as *Shigella dysenteriae* Sh, giving rise in a single growth cycle to normal T2 or T6. The present report attributes the difference between the T* phages and their normal counterparts to an abnormality in the metabolism of uridine diphosphoglucose (UDPG) in strain $B/4_0$, resulting in a defect in the mechanism of glucosylation of phage DNA. The production of phage particles with abnormal DNA in bacteria of this type was reported recently by Eriksen and Szybalski[4] and was discovered independently by Shedlovsky and Brenner.[5]

*Strains, Media, and Methods.*—The strains used were: *E. coli* B, its mutant $B/4_0$ (one of the original isolates[3]), and additional T4-resistant mutants; *E. coli* K-12 strain W3110 (wild type) and its derivative W4597, which behaves as *gal*⁻ because it is defective in uridine diphosphoglucose pyrophosphorylase (UDPG-PPase);[6, 6a, 7] and *S. dysenteriae*, strain Sh.[3] Phages T2, T4, and T6 were from our collection. The media used for most work were tryptone-yeast extract broth and agar and EMB-galactose agar.

UDPG pyrophosphorylase was determined essentially according to Munch-Petersen *et al.*[8] Preparation of enzyme extracts and procedure of enzyme assay have been described by Fukasawa *et al.*[7]

Partial purification of phage DNA was carried out by extraction of phage with water-saturated phenol in the cold, followed by dialysis against $10^{-2}$ *M* Tris-HCl buffer (pH 7.5) containing 0.15 *M* NaCl, and precipitation with ethanol. DNA phosphorus was determined by the microassay of Marinetti.[9] For glucose determination, the DNA was hydrolyzed for 2 hr at 105°C in 1 *N* HCl in a sealed tube, and the liberated glucose was assayed by the enzymic method described by Fukasawa *et al.*[7]

*Results and Discussion.*—Recent experiments[10] have shown that infection of W4597 with phage T2, T4, or T6 gives lysis but fails to produce a crop of normal phage. The lysates have low titers when assayed on *E. coli* strains B or K-12. A relation between these observations and the T* modification produced by *E. coli* $B/4_0$ was suspected when it was observed that *E. coli* $B/4_0$ behaved as *gal*$^-$ when plated on EMB galactose agar. This hypothesis was confirmed by the experiments detailed below, which showed that, in fact, strain $B/4_0$ has a defect in UDPG-PPase, and that T-even phages grown either on this strain or on W4597 behave similarly and are defective in the glucosylation of their DNA.

Table 1 shows the plating efficiency of lysates of T2 and T4 grown on various hosts. It is clear that both strains $B/4_0$ and W4597 release phage that does not grow normally on *E. coli* B or K-12, but grows well on *S. dysenteriae* Sh. After propagation on Sh, the phages return to their normal form. Another strain, *S. dysenteriae* 60, behaves like strain Sh.

TABLE 1

PLATING EFFICIENCY OF LYSATES OF T2 AND T4 GROWN ON VARIOUS HOSTS

| Phage lysate | Bacterial strain on which the phage lysate was prepared | Plating efficiency on: Sh | B | W3110 |
|---|---|---|---|---|
| T2·Sh | Sh | 1.0 | 1.0 | 0.06 |
| T2·B | B | 1.0 | 2.5 | 0.12 |
| T2·K | W3110 | 1.0 | 1.4 | 0.08 |
| T*2·$B/4_0$ | $B/4_0$ | 1.0 | $5 \times 10^{-5}$ | $1 \times 10^{-4}$ |
| T*2·W4597 | W4597 | 1.0 | $5 \times 10^{-3}$ | $2 \times 10^{-4}$ |
| T4·B | B | 1.0 | 1.0 | 1.0 |
| T4·K | W3110 | 1.0 | 1.0 | 1.0 |
| T*4·W4597 | W4597 | 1.0 | $5 \times 10^{-4}$ | $5 \times 10^{-4}$ |
| T2·$B/4_0$·Sh[a] | Sh | 1.0 | 1.0 | 0.05 |
| T4·W4597·Sh[a] | Sh | 1.0 | 1.0 | 0.9 |

[a] These phages were propagated in successive single growth cycles on the two hosts indicated.

The T4 phage released by W4597 will be called T*4, by analogy with T*2 and T*6. Phages T*2 and T*4 adsorb normally to the hosts, *E. coli* B or K-12, on which they fail to multiply.

Table 2 shows that the level of UDPG-PPase in strain $B/4_0$ is less than 2 per cent that in B, and in strain W4597 it is about 10 per cent that of W3110. Several additional isolates of T4-resistant mutants of the $B/4_0$ type, producing T*2 from T2, resembled $B/4_0$ in being *gal*$^-$ and in possessing low levels of UDPG-PPase. Other newly isolated B/4 strains, which did not convert T2 to T*2, were all *gal*$^+$.

Revertants from $B/4_0$ strains capable of utilizing galactose were isolated as dark papillae on EMB-galactose agar. These revertants had lost their resistance

TABLE 2

ACTIVITY OF UDPG PYROPHOSPHORYLASE IN *E. coli* STRAINS

| Strain | Enzyme activity (μmoles TPNH formed per mg protein per hr at 20°C) Expt. 1 | Expt. 2 |
|---|---|---|
| B | 2.8 | 2.9 |
| $B/4_0$ | <0.05 | <0.05 |
| *gal*$^+$ revertant from $B/4_0$ | | 2.9 |
| W3110 | 2.4* | |
| W4597 | 0.3* | |

* Data from Fukasawa *et al.*[5]

to T3, T4, and T7 as well as their ability to modify T2 and T6 to the T* forms. They also had recovered normal UDPG-PPase activity (see Table 2).

Phage T*4 grown on strain W4597, phage T*2 grown on $B/4_0$, as well as normal T4 and T2, were purified, and their DNA was extracted and analyzed. The results, given in Table 3, show that the glucose levels in the T* phage DNA were only about 10 per cent those of the normal controls. In agreement with Eriksen and Szybalski's report,[4] the DNA of T*2 could be separated from that of T2 by density gradient equilibrium centrifugation in $Cs_2SO_4$ (experiments by Dr. David Freifelder).

TABLE 3

GLUCOSE CONTENT OF PHAGE DNA

| Phage | *E. coli* strain on which phage was prepared | Molar ratio [glucose]/[P] |
|---|---|---|
| T2 | B | 0.13 |
| T*2 | $B/4_0$ | 0.01[a] |
| T4 | W3110 | 0.18 |
| T*4 | W4597 | 0.02 |

[a] This value may be too high because the T*2 preparation came from a mass lysate which had a plating efficiency on *E. coli* B higher than that shown in Table 1.

It is remarkable that the reduced glucosylation of phage DNA should, on the one hand, permit the normal assembly of the phage particles and, on the other hand, prevent their successful multiplication in strains of *E. coli* and not in *S. dysenteriae*. The development of phages T*2 and T*4 in the various types of host cells, especially the expression of various phage functions and the fate of the phage DNA in those hosts that do not support phage growth, are currently under investigation.

Cells of strain $B/4_0$ do not adsorb phages T3, T4, and T7. In contrast, cells of strain W4597 adsorb all phages as well as W3110 cells do. Either the receptors for these phages are different in the two *E. coli* strains, B and K-12, or a less complete block of UDPG synthesis in K-12 W4597 allows some of the phage receptors to be produced. In fact, the cell walls of W4597, which contain no galactose, do contain about 10 per cent as much glucose as those of wild-type K-12.[7] Note that W4597 was selected as a *gal*$^-$ mutant, whereas $B/4_0$ was isolated by its resistance to T4. T4-resistant mutants of K-12 defective in UDPG-PPase have not been found. The sugar content of the cell walls of *E. coli* B and $B/4_0$ has not yet been analyzed.

Defects in UDPG-PPase are not the only bacterial genetic blocks that can lead to defective glucosylation of the T-even phage DNA and production of T* phage.

In fact, we have recently produced T*-like phage by growth on two *gal*⁻ mutants of *E. coli* K-12 (isolated by Miss June Rothman), which are unable to utilize glucose as a carbon source but have normal levels of hexokinase, phosphoglucomutase, and UDPG-PPase. The genetic defect in these strains is not in the *gal* operon and probably concerns the ability to synthesize endogenous glucose-6-phosphate.

*Summary.*—Growth of the phages T2 and T4 in *E. coli* mutant strains defective in the enzyme UDPG-pyrophosphorylase yields phage particles of the T* type, whose DNA contains only about 10 per cent as much glucose as that of normal phage. T* phages fail to multiply in their normal *E. coli* hosts, but can reproduce in *S. dysenteriae* strain Sh.

The authors are grateful to Dr. S. E. Luria for suggestions and encouragement during the course of these experiments and for help in preparing the manuscript.

* Aided by grants from the National Science Foundation, G-8808, and the National Institute of Allergy and Infectious Diseases, National Institutes of Health, E3038 (C2).

† Trainee under training grant 2G-602 of the USPHS to the Department of Biology, Massachusetts Institute of Technology.

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## *A CHEMICAL BASIS FOR THE HOST-INDUCED MODIFICATION OF T-EVEN BACTERIOPHAGES*

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*Communicated by S. E. Luria, June 26, 1963*

Bacterial viruses may exist in different forms which are genetically indistinguishable but differ with respect to their ability to multiply in certain host bacteria. The unrestricted form can be propagated on a "permissive" host strain, on which the restricted form is unable to multiply. The conversion of virus particles from one state to the other in the course of a single cycle of growth has been termed host-induced modification. This phenomenon has been studied in a variety of bacterial virus systems[1–3], and it has recently been demonstrated that the host-induced modification of phage λ affects the DNA of the virus.[4, 5]

The experiments presented here reveal the chemical nature of a host-induced modification of T-even bacteriophage.

*Materials and Methods.—Bacterial strains: Escherichia coli* strain B and its derivative BB (Berkeley), and the mutants B/2, B/4, S/6, resistant to T2, T4, and T6, respectively. Strain HfrC *gal*$^-$ (U95) was isolated as a mutant of HfrC, a derivative of *E. coli* K-12, and is unable to utilize galactose as a sole carbon source. Although it has not been analyzed by enzyme assays, this mutant appears to have a defect in the synthesis of uridine diphosphoglucose (UDPG). In several respects it resembles mutant W4597 described by Fukasawa *et al.*[6] : galactose inhibits growth on glycerol, an observation suggesting that the mutant is unable to utilize galactose-1-phosphate.[7] The mutation in U95 is not located in any of the *gal* genes, as shown by the failure of F*gal* and λ*dg* to convert the strain to *gal.*$^+$ In addition, strain U95 does not contain galactose in its cell wall, as shown by its sensitivity to phage C21, a property shared by UDPGal-4-epimeraseless mutants.[8] The mutation in strain U95 appears to be located on the *streptomycin-TL* segment of the chromosome.

*Phage strains:* T2H, T4B*r*$^+$, T6$c_2$.

*Media:* M9 contains, per liter: 5.8 gm $Na_2HPO_4$, 3.0 gm $KH_2PO_4$, 0.5 gm NaCl, 1.0 gm $NH_4Cl$, $10^{-3}$ *M* $MgSO_4$, and 2 gm glucose. Broth contains, per liter: 10 gm Bacto tryptone and 5 gm NaCl.

*Methods:* Phage titers were measured using the agar layer technique[9] on broth agar plates. Purified phage stocks were prepared by infecting logarithmically growing bacteria ($3–4 \times 10^8$/ml) with phage at a multiplicity of 3. After 6 min, the cells were superinfected with the same multiplicity, and incubation was continued for 4 hr. The cultures were then lysed with chloroform, and the phage was purified by two cycles of differential centrifugation. After the first cycle, the phage suspension was treated with RNAase and DNAase (5 μg/ml each). Radioactive phage stocks were prepared in the same way except that the bacteria were infected in M9 medium containing 10 μg/ml $H^3$-hypoxanthine (57 mc/mmole, Radiochemical Centre, Amersham).

*Glucosylation of phage DNA:* Glucosyl transferases, prepared according to the procedure of Kornberg *et al.*,[10] were not purified beyond the streptomycin precipitation step. UDPGal-4-epimerase was obtained as a crude extract of galactose-grown *E. coli* B. Phage DNA was prepared by shaking a purified phage suspension with an equal volume of freshly distilled, water-saturated phenol at room temperature for 2 min. The DNA was precipitated from the aqueous layer with two volumes of 95 % ethanol, collected on a glass stirring rod, and dissolved in 0.01 *M* NaCl in 0.01 *M* Tris buffer pH 7.6. After adjusting the salt concentration to 0.1 *M* NaCl, the DNA was reprecipitated and redissolved as above. Traces of of phenol were removed by repeated ether extraction, and the ether was eliminated by gently bubbling air through the DNA solution. It was found that these DNA preparations contained 180 absorbancy units (at 260 mμ) per mg phosphorus, as measured by the procedure of Allen.[11] UDPGal-$C^{14}$ was prepared by a modification[12] of the procedure of Wiesmeyer and Jordan.[13]

Using the T4 β-glucosyl transferase, each ml of reaction mixture contained: UDPGal-$C^{14}$, 48 mμmoles; $KPO_4$ pH 7.8, 100 μmoles; $MgCl_2$, 25 μmoles; DNA, 10–20 μg; UDPGal-4-epimerase, 500 μg protein; glucosyl transferase, 0.2–0.3 ml. With the T4 α- or the T6 glucosyl transferase, each ml of reaction mixture contained: UDPGal-$C^{14}$, 48 mμmoles; Tris pH 7.8, 100 μmoles; mercaptoenthanol, 33

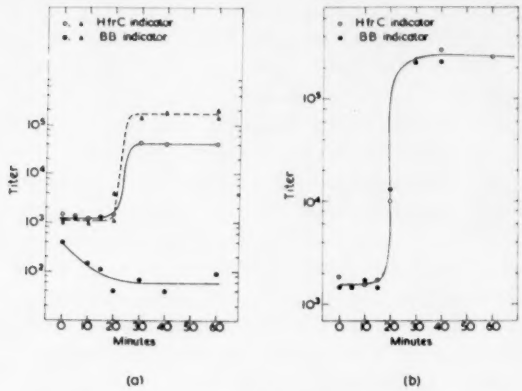


FIG. 1*a*.—One-step growth curve of T6·HfrC on strains HfrC and U95. Broth cultures of strains HfrC (triangles) and U95 (circles) in the exponential phase of growth ($2 \times 10^8$/ml) were centrifuged. The bacteria were resuspended in buffer (M9 medium without glucose) and starved for 15 min at 37°C. L-tryptophan 10 $\mu$g/ml was added, and the cultures were infected with T6·HfrC at a multiplicity of 0.1, as determined by previous titration of the phage on HfrC indicator. After six min of incubation to allow adsorption, the cultures were diluted $10^{-4}$ into broth. During the subsequent incubation, samples were withdrawn and assayed for the number of infective centers on strains HfrC and BB.

FIG. 1*b*.—One-step growth curve of T6·U95 on strain HfrC. Experimental procedure as in Fig. 1*a*.

$\mu$moles; ammonium sulfate, 15 $\mu$moles; DNA 10–20 $\mu$g; UDPGal-4-epimerase, 500 $\mu$g protein; glucosyl transferase, 0.2–0.3 ml. The reaction was initiated by addition of glucosyl transferase. Samples of 0.2 ml were withdrawn and pipetted into 2 ml cold 5% TCA after incubation at 30°C. The samples, after standing in the cold for 10 min, were filtered onto membrane filters, which were washed twice with cold 5% TCA, glued to planchettes, air-dried, and used for radioactivity determination.

*Results.—Host-induced modification of T6 by strain U95:* Initial attempts to grow bacteriophage T6 on strain U95 led to apparently conflicting results. A stock of T6, prepared on *E. coli* strain B, contained $1.4 \times 10^{11}$ plaque-forming units per ml when assayed either on strain B or on strain U95. However, when strain U95 was infected with this phage at a multiplicity of 0.1, no increase in titer, using B as indicator, was detected after 40 min of incubation. Thus, it appeared that T6 could produce plaques when plated on strain U95, but that the phage released from this mutant was unable to grow on strain B.

A comparison of the efficiency of plating (EOP) of purified stocks prepared on strain U95 (T6·U95) and on its parent strain HfrC (T6·HfrC) was made using a

TABLE 1

PLAQUE-FORMING UNITS PER ABSORBANCY 1.0 OF PURIFIED PHAGE STOCKS

| Phage | BB | B | B/2 | HfrC | U95 |
|---|---|---|---|---|---|
| T6·HfrC | $2.5 \times 10^{10}$ | $3 \times 10^{10}$ | $3.3 \times 10^{10}$ | $3.1 \times 10^{10}$ | $2.3 \times 10^{10}$ |
| T6·U95 | $1.3 \times 10^{7}$ | $1.1 \times 10^{7}$ | $1.2 \times 10^{7}$ | $3.5 \times 10^{9}$ | $3.3 \times 10^{9}$ |

Overnight broth cultures of each bacterial strain were diluted into fresh medium and incubated for 3–4 generations until a cell density of $4 \times 10^8$/ml had been reached. Purified phage stocks were diluted and the titers determined on the different indicator strains. The absorbancy at 260 m$\mu$ of each phage stock was measured, and the numbers of plaque-forming units are expressed as titer per unit absorbancy.

variety of plating bacteria. Table 1 demonstrates that, per unit of absorbancy at 260 mμ (a measure of concentration of virus particles), T6·U95 has 0.05–10 per cent EOP of T6·HfrC. More important, it can be seen that T6·U95 has a very low EOP on strains derived from *E. coli* B.

The properties of these phages were examined in the course of a single cycle of growth of U95 and HfrC. It was found (Fig. 1*a*) that one cycle of growth in strain U95 modifies T6·HfrC with respect to its ability to form plaques on strain B, converting it to T6·U95. Furthermore, this modification is lost during a single passage through strain HfrC (Fig. 1*b*). Thus, the properties of these phages resemble in some respects those of the original T* host-induced modification of Luria and Human.[1] The data presented in Figure 1 demonstrate that the rate of growth of these phages is not affected by the host. Although the size of the burst from strain U95, as measured on HfrC indicator, appears to be only one tenth that obtained from strain HfrC, the number of virus particles produced is probably the same in both cases, since purified T6·U95 has an EOP per unit absorbancy on HfrC indicator one tenth that of T6·HfrC.

Bacteriophage T2 and T4 have also been grown on strain U95. The progeny, T2·U95 and T4·U95, exhibit properties similar to those just described for T6·U95. In the case of T2, however, the EOP is difficult to determine on derivatives of *E. coli* K-12, in which even the unmodified phage grow poorly; T4·U95 and T4·HfrC, on the other hand, adsorb slowly to strain U95.

*Properties of T6·U95:* In order to test whether adsorption of T6·U95 to strain BB was normal, a logarithmically growing culture ($5 \times 10^8$/ml) was infected at a multiplicity of about 3 with radioactive phage. At intervals, samples were removed and the number of adsorbed phage determined by filtering onto membrane filters. Since the bacteria are retained by the filter and free phage pass through, adsorption can be measured by determining the amount of radioactivity which is retained on the filter. The rate and amount of adsorption of T6·U95 and T6·HfrC to strain BB were similar. In a control experiment, no adsorption of these phages to strain S/6 was detected. Thus, the modification of T6·U95 does not affect adsorption to the nonpermissive host.

Upon testing the viability of cells which have been infected with T6·U95, it was found that these phages kill bacteria in which they are unable to multiply. These experiments further revealed that the apparent titer of a stock of T6·U95 increases with increasing multiplicity of infection and is also, to some extent, dependent upon the physiological state of the infected bacteria: the restriction of T6·U95 multiplication is more pronounced during the exponential than during the stationary phase of bacterial growth.

It has been shown by Dussoix and Arber[5] that a host-induced modification of phage λ results in the destruction of its DNA after infection into a nonpermissive host. To test whether a similar destruction occurs in BB infected with T6·U95, samples from cultures infected with DNA-labeled phage were withdrawn, chilled, and precipitated with cold TCA (5 per cent). These samples were filtered, washed with cold TCA, and the precipitated radioactivity was determined. No loss of counts (sufficient to account for the low EOP) was detected, an indication that there is no extensive destruction of this DNA, although degradation to acid-precipitable fragments cannot be excluded.

*Glucosylation:* It has been shown that the hydroxymethylcytosine (HMC) residues of T-even phage are substituted with one (or two) glucose molecules.[14–17] The pattern of glucosylation is specific for each phage type. Furthermore, it is known that the glucosylation of these pyrimidines proceeds by a transfer of glucose from UDPG to the hydroxymethyl group of HMC in DNA.[10, 18] Since strain U95 appears to be defective in UDPG biosynthesis, we have investigated whether the DNA of T6·U95 contains glucosylated or unglucosylated DNA. DNA from purified stocks of T6·HfrC and T6·U95 was prepared. Equal amounts of DNA were assayed for hexose content by means of the anthrone reaction.[19] The results of this experiment indicated that the hexose content for T6·U95 DNA is about 2 per cent that of T6·HfrC. This is only a rough estimate, and the actual glucose content in T6·U95 may actually be higher or lower.

We have tested the capacity of these DNA preparations to accept glucose in the *in vitro* glucosylating system described by Kornberg.[10] It was found that no glucose could be added to the DNA of T4·HfrC or T6·HfrC. In contrast, the DNA derived from T4·U95 did accept glucose, as illustrated in Figure 2. In these experiments, carried out most extensively with the T4 glucosylating system, it was found that about 90 per cent of the theoretically available residues could be substituted. Thus, in T6, 75 per cent of the HMC bases are normally glucosylated, and we have found that 63 per cent of the HMC residues can be substituted using DNA from T6·U95. In T4, 100 per cent of the HMC is normally glucosylated; using T4·U95 DNA we found that 80–100 per cent of the HMC residues can be glucosylated by either the $\alpha$- or the $\beta$- T4 glucosyl transferase. Similar results have been obtained by Josse and Kornberg using HMC-DNA synthesized *in vitro*.[20]

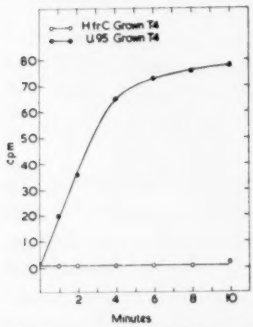


FIG. 2.—Glucosylation of DNA from T4·HfrC and T4·U95. The experimental procedure is presented under *Materials and Methods*.

*Discussion.*—These experiments demonstrate that the DNA of T-even phages which have been propagated on strain U95 contains little or no glucose. Such phages will form plaques with relatively high efficiency on U95 or HfrC, yielding what is apparently normal T6. The presence or absence of glucose does not affect the nature of the genetic information contained in the DNA, but only the capacity of the phage to grow in certain hosts. On strain B, growth of nonglucosylated phage is restricted, and this might be due to the presence in this bacterial host of an enzyme which degraded such DNA. We have been unable, however, to demonstrate extensive degradation of the DNA such as was found by Dussoix and Arber[5] for host-modified $\lambda$. If the restricting enzyme makes only a few breaks in the modified DNA, our failure to find extensive degradation may arise from the resistance of HMC-DNA to subsequent hydrolysis by other enzymes which attack cytosine-DNA. The presence of glucose on the HMC bases might inhibit the action of the restricting enzyme. There may be many such restricting enzymes with different specificities, and the varying patterns of glucosylation in the different T phages may reflect the range of such specificities. For example, although T2 is not restricted on strain B despite the fact that only 75 per cent of its HMC bases are glucosylated, other bacterial

strains may exist which restrict T2, but not T4, which is fully glucosylated. In fact, *E. coli* K-12 may be one such strain since T2 grows with low efficiency in this host.[21]

In a reinvestigation of the T* modification, Hattman and Fukasawa[22] have shown that the modifying host, strain $B/4_0$, is defective in UDPG pyrophosphorylase, and that strain W4597, also defective in this enzyme,[6] produces similarly modified phage. Revertants of strain $B/4_0$ not only regain enzyme activity, but also lose the ability to modify T2 and T6. Thus, it appears that the three mutants, $B/4_0$, W4597, and U95, produce a class of phage modifications of T-even phage due to unglucosylated HMC-DNA. Whether strain U95 has the same genetic defect as $B/4_0$ and W4597 has not yet been ascertained; nor has it been tested whether the phages grown on these various bacteria behave identically with respect to host range.

In view of the recent demonstrations[23, 24] that DNA may contain bases other than cytosine, thymine, adenine, and guanine, it seems likely that the chemical basis for host-induced modification, in general, may be analogous to that described here. For example, host-controlled variations in methylation of certain bases could lead to the production of biologically modified viruses.

*Summary.*—A mutant of *E. coli* HfrC which is defective in UDPG biosynthesis has been shown to modify T-even phage. The biological expression of this modification consists of the failure to grow in derivatives of *E. coli* strain B. A chemical basis for this modification has been found in that the DNA of these phages contains little or no glucose.

* Postdoctoral fellow of The National Foundation.

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# *PRIMITIVE NERVOUS SYSTEMS**

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*Communicated by G. E. Hutchinson, June 17, 1963*

For nearly fifty years the dominant and accepted theory of the evolution of the nervous system has been that of G. H. Parker, developed over the course of the decade from 1909 to 1919 and summarized in his now classic book *The Elementary Nervous System*.[1] Like many successful theories, it was an oversimplification of contemporary knowledge presented in a forceful and stimulating manner. In essence, Parker viewed the initial evolution of the nervous system as involving three successive phylogenetic stages. At first there were only "independent effectors," represented today (in Parker's view) by sponge myocytes or coelenterate nematocysts. Secondly, receptor cells evolved from undifferentiated epithelium adjacent to the muscle cells. "The most primitive nerve cell from the standpoint of animal phylogeny is the sense-cell, or receptive cell, such as occurs in the sensory epithelium of the coelenterates."[1] Finally, "protoneurons" evolved between receptor and effector to give rise eventually to the reflex triad of receptor, adjustor, and effector.

Parker's was not the first theory to be expounded. A generation before him Kleinenberg[2] had used his discovery of the "neuromuscular cell" of *Hydra* to support the theory that the reflex triad originated from the division, in evolution, of what had once been a single cell. But the Hertwigs[2] identified the "neuromuscular cell" as an epitheliomuscular cell, and, invoking the biogenetic law, suggested instead a simultaneous evolution of nerve and muscle cells from separate epithelial cells, as they are formed in coelenterate development. Until Parker's theory was advanced, the Hertwigs' theory was the one generally accepted; it was rapidly replaced, however, by the strength of Parker's concept of the independent effector stage, exemplified by the sponges.

Since a great deal of what is known about nervous systems has been learned since the publication of Parker's book, in addition to a modest revival of interest in the nervous system of coelenterates, led by Pantin, it is noteworthy that the independent effector theory continues to be generally accepted. Neurophysiologists such as Bishop[3] and Grundfest[4] have emphasized that graded responses unknown in Parker's day certainly antedate the all-or-none action potential of the nerve cell axon, while the terminal secretory activity of the synaptic transmitter would also be expected to have evolved as a separate step. But neither question the fundamental sequence in Parker's theory that the independent effector evolved first and that then, in the next "brief and logical step,"[3] a special sensory cell evolved to depolarize the adjacent contractile unit at the "first synapse."[3]

Pantin, whose approach has been that of the coelenterate biologist rather than that of the neurophysiologist, has been more critical[5] of Parker's theory. Rather than being concerned with the evolution of certain physiological processes, he has considered the entire biology of the postulated ancestral forms, for "the metazoan behaviour machine from its origin. . .must have involved the structure of the whole animal, and it must have been complex enough and organized enough to meet all the varied requirements of behaviour."[5] Just as in coelenterates today, the effector

of the prenervous animal was not a single cell acting individually, but an entire muscle sheet. Innervation of a single muscle cell by a receptor cell could not initiate any meaningful response without conduction to all units of a response field.

As Sherrington long ago pointed out,[6] a single protoneuron "conductor" cannot function as the "common path" necessary for nervous system integration. Inputs of many receptors must merge on common "coordinators" before integration is achieved.[7] *Integration is as fundamental as conduction to any nervous system.* An organism at the phylogenetic stage showing isolated Parkerian triads, neither integrating nor conducting to more than a single effector, would have no advantage over a previous stage without such triads, and thus is evolutionally implausible.

There are, moreover, further objections to Parker's theory than solely the inadequacy of isolated triads of receptor cells, protoneurons, and effector cells. The importance of inherent rhythmic behavior is becoming ever more apparent,[8] in part because of the investigations of actinian behavior carried out by Batham and Pantin.[9] As a result of these findings, as well as the realization that metazoan behavior is not organized cellularly, but with tissues such as muscle sheets as its units, Pantin has suggested such important modifications of Parker's theory that his views should be considered a new hypothesis. He suggests[5] that the nerve-net has originated as a supplement to a conducting and integrating muscle sheet. The nerve-net's initial function was to permit the development of specialized through-conduction tracts, allowing the evolution of such reflexes as the *Calliactis* sphincter closure. Spontaneous activity, either in the effectors (the muscle sheet) or in the "conducting nervous system itself,"[5] can supplement direct reflexes. Thus, Pantin sees the nervous system evolving (without specifying in detail from whence it came) in an integrated organism with a well-developed muscle sheet effector system. Sensory stimuli would affect this "system of independent effectors."[5] Pantin's hypothesis is a distinct improvement on Parker's, as it provides for endogenous activity within the primitive nervous system as well as meeting the other objections discussed above.

Both Parker[1] and Pantin[5] agree that it is inconceivable that any nervous system could evolve prior to the evolution of muscles or other effectors (cilia, gland cells, etc.). Admitting this, it does not necessarily follow that these primitive metazoans had to have receptors before they could evolve conductors, or, as the protoneurons of Parker were called by the Hertwigs, "ganglion cells." As soon as the possibility is admitted that neuronal activity can arise endogenously without the involvement of external energy stimulation, it becomes an open question as to whether Parker's (and Pantin's) phylogenetic sequence of independent effector, receptor-effector, and finally receptor-adjustor-effector is correct or whether receptors evolved subsequent to ganglion cells.

We propose that individual protomyocytes first evolved into assemblages of independent contractile cells, permitting more extensive movements than those resulting from contractions of individual myocytes. Certain of these cells became endogenous activity centers, or *pacemakers*, by developing unstable specialized membrane areas capable of active depolarization. Such local pacemakers synchronized contractions of adjacent cells by passive depolarization spread affecting the contractile mechanism, perhaps utilizing intercellular bridges. Groups of muscle cells responding to pacemakers would permit the evolution of recurrent

feeding movements. Differentiation of these two cell types would have thus proceeded together, with what was to become muscle gradually becoming specialized for contraction and what was to become nerve becoming specialized for activity initiation. Initially both would become specialized for passive conduction of depolarization. The specialization of the nerve cell for the conduction rather than the repetitive initiation of activity is seen as a secondary development in the evolution of neurons.

The new hypothesis of the origin of the nervous system presented here is like that of the previous theories in that it, too, is the result of investigation of the coelenterate nervous system, although mainly of scyphomedusae and *Hydra* rather than the actinians which have been Pantin's prime concern. The striking regional autonomy found in many coelenterates—e.g., separate spontaneously active stalk, proximal tentacles (individually and collectively), distal tentacles, and manubrium in the *Corymorpha* polyp[10]—suggests that pacemaker loci at first did not integrate the entire organism as a unit. Rather, physiological unification by interconnected nerve-nets could have come later, after nondecremental all-or-none impulses had been achieved. It is worthwhile suggesting that such nervous unification coincided with the completion of individuation of the protocoelenterate from a colonial ancestor. Certainly analogous examples of incomplete individuation occur today within such colonial forms as *Physalia* and *Renilla*.

When such local action systems as tentacles or manubrium, each with their local pacemaker loci, were linked together, some of these pacemakers would become specialized for over-all control of the organism, whereas others would become subordinate centers controlling specific activities. Such a development would mark the achievement of the polarized synapse, as occurs in the scyphomedusan marginal ganglion. A hierarchy of pacemakers would emerge. The activity of centers controlling effectors indirectly through subordinate regional pacemakers would then become concealed, or cryptic, and such nervous activity could proceed to evolve without being directly tied to effector responses.

At the same time the nervous system could develop increased and differential sensitivities to various external stimuli, and thus integrate recurring internal activities with rhythmic external events; but even prior to this time pacemaker cells would have been influenced by impinging stimuli from external sources. Such external modulation of an existing output pattern would make them more sensitive to weak external stimuli; the same principle occurs in nearly all afferent pathways in higher animals. There is evidence that coelenterate neurons are still directly sensitive to rather short wavelength visible light,[11] a property shown by many of the lower invertebrates.

The further evolution of the nervous system would involve the development of through-conducting tracts for specialized reflexes, as Pantin has outlined,[5] the appearance of concentrations of nerve cells as ganglia or nerve rings, and the evolution of concentrations of receptors into sense organs in association with these ganglia. Such a phylogenetic series can be seen within the coelenterates, culminating in the elaborate rhopalia of the cubomedusae. There is a tendency toward reduction in the number of ganglia, shown also by the ctenophores, that may indicate how the cephalic dominance of the bilateria was achieved. Patterned activity from interacting pacemakers would continue to dominate such central nervous

systems, evolving eventually into the brain waves of the higher invertebrates.[12]

It can be seen then that this hypothesis of the primitive nervous system departs markedly from that of Parker and significantly from that of Pantin. It marks somewhat of a return toward the views of the Hertwigs of eighty-odd years ago. Nerves and muscles have evolved in parallel from the first differentiation of either, the former first developing endogenous rhythmic activity and subsequently synapses and through-conduction tracts. Sensory reception was primarily a subsidiary function of the "ganglion cells" and only with the development of hierarchies of pacemakers did specialized sensory receptors appear.

How well does this new hypothesis summarize our current understanding of the existing primitive nervous systems of the lower invertebrates?

Prosser *et al.*[13] have recently shown that depolarization across cell membranes, passively or actively, probably does not occur in a variety of sponges. This not only casts further doubts on the view that a nervous system occurs in this phylum, but also differentiates their contractile mechanism from the conducting mechanism of coelenterates, since potassium-enriched sea water solutions which block both nerve-nets in scyphomedusae do not affect sponge myocytes.

Information from the coelenterates can be divided into three categories: histological, behavioral, and that obtained from direct neurophysiological recording. There are no coelenterates as yet where anything approaching a complete picture of nerve function has been achieved; only bits of information are available.

There is clear histological evidence for the presence of two overlying nerve-nets occupying the same epithelium, in several classes of coelenterates. In *Velella* one of these nets appears to be a syncytium,[14] but in several species of scyphomedusae each nerve-net (the diffuse and the giant fiber) consists of two-dimensional feltings.[15] So-called "sense-cells," i.e., neurons with short flagella, occur only in the diffuse nerve-net, but there is no evidence that this nerve-net is more sensitive to external stimuli in the subumbrellar region where both occur. Elsewhere in the phylum, there is little to suggest such superimposed nets, although "sense cells" seem to form local assemblages in the margins between column and mesenteries in sea anemones.[16]

Concentrations of neurons occur in medusae but are unknown in polyps. As they contain the pacemakers responsible for the swimming beat and are associated with nearly all of the sense organs found in the phylum, their absence from polypoid stages might suggest that the latter have less nervous activity, but direct physiological evidence refutes this conclusion. The isolated column of the hydroid polyp *Corymorpha palma* shows a negative rheotropism[10] just as does a statocyst-equipped hydromedusan, and many coelenterates show light responses without demonstrable pigment spots or ocelli. The absence of such "ganglia" does not preclude endogenous activity any more than does the absence of recognizable receptors preclude sensitivity to external stimuli.

Recurring behavior patterns in a wide variety of coelenterates are important evidence for rhythmic nervous activity. Such activity may be regular or irregular, continuous or occasional, almost too fast to be perceived or far too slow to be perceived. For example, the rhythmic activity of *Hydra* was pointed out by Jennings,[17] but its irregularity has caused most observers to ignore it. This variability of response is actually an accurate measure of internal activity, for as Mast pointed out fifty years ago: "Everyone who has ever attempted observations on the be-

havior of organisms with precise methods, knows that variability even in the lower forms under constant external conditions is one of the striking characteristics in reactions. There are internal as well as external factors involved in determining what the organism is to do."[18] So it is with *Hydra*.

The range in rates of activity is remarkable. Some coelenterate rhythmic activities are shown in Table 1. It seems scarcely credible that the same mechanism

TABLE 1

RHYTHMIC ACTIVITIES IN VARIOUS COELENTERATES

| Organism | Authority | Activity | Rate per minute |
|---|---|---|---|
| Many colonial hydroids: *Campanularia, Cordylophora, Obelia, Pennaria*, etc. | 24 | Back and forth movements in stolons; pulsations behind growing tip; hydranth twitches | 0.05–0.35 |
| *Cordylophora* | 21 | Repetitive firing after stimulation | 60–300 |
| *Hydra* | 11, 17 | Contraction bursts | 0.05–0.2 |
| | 11 | Rhythmic potentials | 1–7 |
| | 11 | Attachment bursts | 10–20 |
| *Corymorpha* | 1,10 | Feeding activity | 0.25–0.33 |
| *Porpita* | 14 | Feeding activity | 2 |
| *Tubularia* | 21 | Feeding activity | 2 |
| | 21 | Potentials | 2–45 |
| | 21 | Potentials in bursts | 300 |
| *Physalia* | 1, 14 | Raising fishing tentacles | 1 |
| Many hydromedusae | 1, 15, 17 | Velum contractions | 6–120 |
| Many scyphomedusae | 15 | Subumbrellar muscle contractions | 6–80 |
| Actinians: | | | |
| *Calliactis* | 16, 20, 23 | Circular muscle | 0.07–0.1 |
| *Metridium* | 9 | Circular muscle | 0.04 |
| | 7 | Phases | 0.0002–0.005 |
| *Stomphia* | 19 | Basal disk rings | 0.03 |
| | 19 | Parieto-basilar contractions, "swimming" | 20 |
| *Renilla* | 25 | Repetitive afterdischarges after stimulation | 50 |

can cause repetition rates of 200, 2, 0.02, and 0.0002 per minute, and perhaps for this reason, as much as for the negative histological evidence, Pantin has suggested that the slower rhythms are myogenic rather than nervous. However, in *Hydra* at least even the slow contraction rhythm (0.05–0.2 per minute) is indirectly controlled by the "rhythmic potential" pacemakers.[11] Slow actinian rhythmic activity could be nervously controlled by such an indirect mechanism. Occasionally there is evidence for a slow rhythmic activity, as the extremely regular contractions (0.03 per minute) of the basal disk ring preparation, and a rapid rhythmic activity (parietobasilar contractions up to 20 per min) in the same organism, in this case the "swimming" sea anemone *Stomphia*.[19]

Another behavioral clue to pacemaker activity, in addition to rhythmicity, is a patterned output. This is also demonstrated by *Hydra*[11] where each over-all contraction consists of a "contraction burst" or 7–12 or more individual contractions, arranged in a characteristic time sequence. The patterns vary according to the physiological state and species of polyp. Pacemaker location can be shown by the specific blocking effect of localized hypostomal illumination. Light inhibition becomes progressively more difficult, until finally illumination is unable to halt the contraction burst. Comparable patterned behavior is also seen in the feeding reflex of *Porpita*[14] and in the intermittent swimming patterns of many hydromedusae.[17] Horridge has shown how stable and rapid rhythms can be achieved by the multiple interactions of all eight marginal ganglion pacemakers in scyphome-

dusae;[15] the same result—the increased rate of feeding movements in an intact polyp as compared to either isolated stalk or isolated hydranth—is evidence for multiple pacemaker control in *Corymorpha*.[10]

The most distinctive feature of coelenterate neurophysiology is *facilitation*, first shown by Pantin using the *Calliactis* sphincter muscle.[20] Facilitation means that a single nerve impulse does not cause a contraction, but that a second nerve impulse following the first causes a muscle contraction whose strength depends on the time interval between the first and second impulses. Pantin deduced that a similar mechanism, interneuronal facilitation, occurred between certain neurons, as in conduction around the sea anemone's oral disk. Facilitation also occurs at the scyphomedusan neuromuscular junction (although incompletely), within the nerve-net connecting individuals of colonial corals, and almost everywhere else within the phylum's neuromuscular system.

It was fortunate that the forms used in Pantin's classic studies do not show tendencies for spontaneous afterdischarges; with rare exceptions,[9] only when the electric stimulus is increased manyfold do two or more nerve impulses occur after a single stimulus. In other forms afterdischarges are more common. By recording its nerve impulses directly Josephson has shown how repetitive firing leads to graded conduction spread along a *Cordylophora* colony,[21] and thus, incidentally, validated the belief that direct recording of coelenterate nervous activity must be used to confirm indirect findings. It is now inferred that the same mechanism holds for other colonial forms, including the corals. The induced impulses do not necessarily arise from the site originally responding to the stimulus; thus, potential impulse initiation sites occur within the nerve-net, i.e., latent pacemakers, whose responsiveness may fluctuate in a complex manner. Subsequently, Josephson recorded the spontaneous nervous activity of the hydroid polyp *Tubularia*,[21] which shows a disconcerting variety of pacemaker sites, patterned bursts, and interactions that belie the seemingly stodgy behavior of this animal. However, this behavioral simplicity is probably erroneous, for *Tubularia* is rather closely related to both *Corymorpha* and *Porpita*, whose behavior has been mentioned above. Parker investigated *Corymorpha* because he was looking for a "simple" coelenterate to fill the gap between sponge and sea anemone, but after studying it he admitted that its behavior was far from "simple." Yet both *Tubularia* and *Corymorpha* are rather primitive hydroids.[22]

Taking *Tubularia*,[21] *Corymorpha*,[10] and *Porpita*[14] together, it can be seen that the first shows rapid, cryptic nervous activity which is both spontaneous and rhythmic, as well as intermittent pacemaker bursts. The latter genera show intermittent behavior strongly suggestive of pacemaker control, and in *Porpita* the feeding reflex consists of rapidly repeating coordinated tentacle jerks as well. It seems very likely that all three have similar nervous and behavioral mechanisms, appropriately modified to their own habits.

In *Hydra* it has been possible to study both behavior and the coordinating system directly, at the same time and for extended periods.[11] But our knowledge of its nervous structure is still so inadequate that it is impossible to be sure that what has been recorded has been nervous activity alone; the endodermal muscle sheet might be a conducting net as well as an effector. Nevertheless, tentatively and primarily for convenience, we will use the term *nervous* here. It is obvious that

this question must be resolved in deciding whether the nervous system is a primary or secondary development.

The basic nervous activity in *Hydra* consists of spontaneous "rhythmic potentials"[11] originating from any one of a number of potential pacemakers in the lower column and stalk. These pulses are conducted without decrement throughout the column (distances in excess of 2 cm in the large *H. piradi*) without causing any discernible muscle responses. Their rhythm may be quite irregular or it may be very constant, while their rate also varies. Rhythmic potential pacemakers are directly sensitive to light, so that at dawn there is a nearly twofold increase in the rhythmic potential frequency which only slowly declines during the day.[11] The behavior patterns of the polyps show parallel changes.

The main action system of *Hydra*, the ectodermal longitudinal muscles, effects the periodically recurring contraction burst. With a patterned sequence of coordinated powerful contractions, the polyp is pulled down into a tight ball. Each contraction is preceded by an impulse originating from the hypostome region and conducted down the column by a nerve-net separate from that of the rhythmic potentials. Re-extension appears to be passive. Localized light stimulation can halt such contraction bursts by acting directly on the burst pacemaker.

The dark-adapted *Hydra* shows a characteristic response to light involving the tentacles, column, and contraction burst frequency. This response is mediated through the rhythmic potential system, since it will occur in an identical manner when only the rhythmic potential pacemakers are illuminated.[11] This is taken as proof of the dominant role of the rhythmic potential system. It is evident that this system is a cryptic coordinating network, primarily endogenously active but susceptible to external (light; strong mechanical), proprioceptive, and enteroceptive stimuli. It controls the animal's activity by its control of subordinate but semi-autonomous pacemakers which, in turn, control effector systems. The rhythmic potential system is the machinery for spontaneous activity.

In scyphomedusae there is no hidden rhythmic pacemaker activity in the diffuse nerve-net, probably because of the rhythmic activity of the giant fiber nerve-net innervating the subumbrellar muscles. Diffuse nerve-net impulses act on the marginal ganglion pacemakers which, in turn, initiate impulses in the giant fiber nerve-net. What is noteworthy in this advanced nervous system is the dearth of afferent stimuli. Spontaneity occurs in its absence. The specialized sense organs associated with the marginal ganglia—the rhopalia—do not appear to originate nerve impulses in either of the two through-conducting nerve-nets. There is nothing to suggest a primary role for sensory receptors in the evolution of these nervous systems.

In the forty-odd years since Parker's book appeared there has been a renewed appreciation of the complexity and organismic unity of the lower animals. We now recognize the existence of spontaneous activity, of rhythmic behavior patterns of considerable complexity, of the interactions of multiple pacemaker loci existing within several superimposed conducting systems, of cryptic nervous activity; all these changes of view allow the biologist to account for the behavior of entire organisms, with its phases and diversity, without invoking purposefulness. For example, a cryptic pacemaker system could provide the physiological machinery, the chronometry, for the ". . . variety of more or less periodic active systems . . ."[9]

which occur in coelenterates. Parker's theory is no longer adequate and must be replaced, but whatever hypothesis is followed, the totality of the organism's behavior must be explained in developing a comprehensible view of the nervous system of primitive animals and its evolution.

The author wishes to thank Drs. E. D. Hanson and C. P. Mangum and Mr. F. M. Williams for their help with this manuscript.

* Supported in part by research grant G-14574 from the National Science Foundation.

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# *DISSOCIATION OF RABBIT GAMMA GLOBULIN INTO SUBUNITS BY REDUCTION AND ACIDIFICATION**

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*Communicated by H. E. Carter, June 24, 1963*

The work of Edelman and collaborators[1–3] and of other investigators[4–6] has demonstrated that 6.5 S gamma globulin molecules of various species consist of several polypeptide chains. Recent experiments of Fleischman, Pain, and Porter[7, 8] and of Marler and Tanford[9] indicate the presence in rabbit gamma globulin of two chains of molecular weight of 55,000–60,000 each, and two of molecular weight 20,000–25,000. Fleischman *et al.* denote the two types of polypeptide chains as "A" and "B," respectively, corresponding to the "H" and "L" chains of human gamma globulin, described by Edelman and associates. On the basis of experiments with horse antibody, Porter has concluded that the "A chains" contain the active combining sites of the antibody molecule.[8] Utsumi and Karush,[10] and Jaquet, Bloom, and Cebra,[11] have obtained active subunits of rabbit antibody by reduction and treatment with detergent.

It was recently demonstrated that the disulfide bond which links the univalent fragments of rabbit antibody after treatment with pepsin can readily be reduced prior to enzymatic digestion.[12] Complete reduction of this bond was observed when a total of 5–6 $-SH$ groups had been liberated, and was characterized by the formation of univalent, 3.5 S fragments upon subsequent treatment with pepsin. (Peptic digestion of the unreduced antibody molecule yields a bivalent, 5 S fragment which is split into 3.5 S fragments by reduction of one labile disulfide bond.[13, 14]) We have now found that acidification of a solution of gamma globulin in 0.1 *M* NaCl, after reduction of 2–3 disulfide bonds, results in a decrease in molecular weight to approximately half that of the original molecule. The product forms one symmetrical peak in the ultracentrifuge. The results suggest that two subunits, approximately equal in size, are liberated. When the pH is then raised to neutrality, varying proportions of two major components, sedimenting with velocities of approximately 4 S and 6 S, are observed. The latter value is almost identical with that of untreated gamma globulin, which suggests that spontaneous recombination occurs at neutral pH. There is no appreciable formation of insoluble material, and a large fraction of the specific antibody activity of an antihapten antibody was retained.

*Materials.*—Methods used to obtain rabbit antisera against chicken ovalbumin or the *p*-azobenzoate haptenic group, and for the preparation of gamma globulin fractions, have been described elsewhere.[15–18] Immunoelectrophoresis of each gamma globulin preparation with sheep anti-rabbit serum showed only a single band, characteristic of gamma globulin. Chicken ovalbumin, twice crystallized, was obtained from the Pentex Company. 2-Mercaptoethylamine hydrochloride (MEA) was purchased from the California Corporation for Biochemical Research. *p*-Nitrobenzoic acid labeled with $C^{14}$ in the carboxyl group (1.2 mc/mmole) was obtained from the New England Nuclear Corporation, and recrystallized. The radiochemical purity, estimated by the isotope dilution method, was found to be greater than 97%.

*Methods.*—Measurements of the capacity of anti-*p*-azobenzoate preparations to bind *p*-nitrobenzoate-$C^{14}$ were made by the method of equilibrium dialysis at 5.0°C, in saline-borate buffer, pH 8.0, ionic strength 0.16.[19] Samples to be compared were dialyzed against a common outer solution.

Measurements of radioactivity per unit volume of the inner and outer solutions were made after dialysis to equilibrium. Samples were evaporated to dryness in transparent cellulose acetate planchets, placed in cylindrical glass vials filled with 15 ml of redistilled toluene containing 1.5 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and 60 mg of 2,5-diphenyloxazole (PPO), and counted at −20°C in a Packard Tri-Carb liquid scintillation counter. Protein was added to the outer solution prior to evaporation to equalize the amounts present in the samples.

Molecular weights were determined by the methods of sedimentation diffusion or sedimentation equilibrium in a short column. Diffusion constants were determined in a synthetic boundary cell in the ultracentrifuge at 9,341 rpm, by the method of Ehrenberg,[20] and calculated from graphs of $1/y^2_{max}$ against time, where $y_{max}$ is the height of the peak in the schlieren pattern. The sedimentation equilibrium experiment was carried out at 20°C and 12,590 rpm; a 1.7 mm column in a filled epoxy double sector cell was used. Sedimentation velocities were determined at 59,780 rpm and 20° ± 0.2°C in 12 mm, 2°, single or double sector Kel-F cells. In correcting values to $s_{20,w}$ the partial specific volume was taken as that of untreated gamma globulin, 0.745.[21, 22] Except as indicated, the protein concentration was 5–8 mg/ml.

Sulfhydryl groups were estimated essentially as described by Boyer.[23] Details are given in reference 15. After reduction and removal of the reducing agent, samples were treated with an excess of *p*-chloromercuribenzoic acid (CMB) and back-titrated in the presence of 0.5% sodium lauryl sulfate with a freshly prepared solution of recrystallized L-cysteine.

Gamma globulin preparations were reduced with 2-mercaptoethylamine hydrochloride (MEA) at pH 5 and 37°C for 75 min, then passed through a column of IR-120 cation-exchange resin at pH 5.0 in the cold room to remove the MEA. Details of the methods have been described elsewhere.[15] The eluate was neutralized, and excess CMB was added immediately. In some experiments CMB was added just before neutralization.

*Results.—Reduction of rabbit anti-ovalbumin gamma globulin, followed by acidification:* Figure 1 shows sedimentation patterns obtained after reduction of gamma globulin (preparation A) with decreasing concentrations of MEA, and subsequent adjustment of the pH to 2.5 with 1 *M* HCl. Samples were dialyzed against 0.1 *M* sodium chloride at neutral pH before acidification. With 0.5 or 0.1 *M* MEA, the product, at pH 2.5, migrated almost entirely as a single peak ($s_{20,w}$ = 3.4 S in each case—Figs. 1*A* and 1*B*). Small amounts of a faster-moving component were present. At the same pH and salt concentration, the $s_{20,w}$ value of the unreduced gamma globulin was 5.0 S (Fig. 1*E*). When 0.05 *M* or 0.01 *M* MEA was used for the reduction, incomplete transformation to the product having a lower sedimentation co-

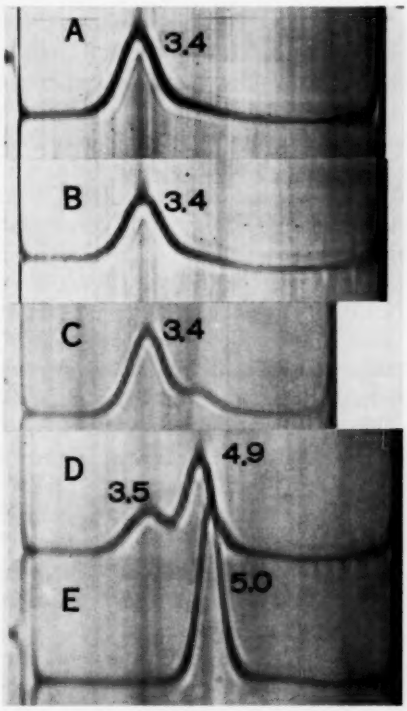


FIG. 1.—Photographs of schlieren patterns taken in a Spinco Model E ultracentrifuge after 80 min at 59,780 rpm. Sedimentation is from left to right. Samples in 0.1 *M* NaCl plus HCl, pH 2.5. (*A*) Gamma globulin reduced with 0.5 *M* MEA, 9.3 —SH groups per molecule; (*B*) 0.1 *M* MEA, 9.1—SH groups; (*C*) 0.05 *M* MEA, 5.2 —SH groups; (*D*) 0.01 *M* MEA, 1.8 —SH groups; (*E*) unreduced gamma globulin, 0.7 —SH groups. Details are in the text. The numerals in the figure are $s_{20,w}$ values.

efficient was observed (Figs. 1*C* and 1*D*); this was especially pronounced for the sample treated with 0.01 *M* MEA. The sedimentation velocity of the faster component in each case was very similar to that of the unreduced control (Fig. 1*E*). The number of free —SH groups per molecule of protein, for each preparation, is indicated in the legend of Figure 1. The value, 0.7 —SH per molecule, was obtained for the unreduced control, which was similarly incubated at pH 5.0 and passed through a column of IR-120 resin. This value, when subtracted from each of the other values, gives the number of —SH groups liberated by reduction. The data indicate that 2–3 disulfide bonds must be reduced for complete transformation to the slower-moving form. The value, 9.3 —SH groups per molecule, obtained after reduction with 0.5 *M* MEA, was 2–3 —SH groups lower than that observed in similar experiments with other preparations.

At neutral pH the $s_{20,w}$ value of the sample treated with 0.5 *M* MEA (6.5 S) was similar to that of the unreduced gamma globulin.

These data suggest the possibility that the reduction and acidification result in a decrease in molecular weight, and that the samples reduced with the lower concentrations of MEA (0.01 *M* or 0.05 *M*) exhibit 2 components in the ultracentrifuge because of inadequate reduction of disulfide bonds.

*Molecular weight of the 3.4 S component:* A 500 mg sample of another preparation of antiovalbumin gamma globulin (preparation B) was reduced with 0.1 *M* MEA, treated with CMB, and dialyzed against cold 0.1 *M* NaCl at neutral pH. A total of 8.9 —SH groups per molecule of protein was liberated in this procedure. A portion was concentrated by pervaporation to a protein concentration of 30 mg/ml, then dialyzed against another 4 liter portion of the same salt solution. At pH 7, the $s_{20,w}$ value was 6.2 S, which is close to that of the untreated gamma globulin (6.1 S). The pH of a 30 mg portion was lowered to 2.5 with 1 *M* HCl. The $s_{20,w}$ value of the major component at this pH was 3.4 S. An appreciable amount of faster-moving material was also present. At the same pH and salt concentration the $s_{20,w}$ value of the unreduced gamma globulin was 4.6 S.

In an effort to separate the major 3.4 S protein from the faster-moving components, 30 mg in 1 ml was chromatographed on a 110 $cm^3$ column of Sephadex G-200, equilibrated with 0.1 *M* NaCl containing sufficient HCl to lower the pH to 2.5. Two thirds of the protein, eluted first from the column, was discarded. The last third was used for determinations of molecular weight, following dialysis against cold 0.1 *M* NaCl, pH 2.5. The $s_{20,w}$ value was the same as that of the major component observed prior to passage through Sephadex (3.4 S). The sedimentation velocity was measured in a double-sector cell, with a portion of the outer solution of the dialysis present in the other compartment of the cell. The gradient curve obtained in this experiment was analyzed by subtracting from the leading side of the principal (3.4 S) boundary the mirror image of the trailing side. This resolved the "shoulder" into a small peak, with area corresponding to 10% of the whole, and sedimentation coefficient of approximately 5 S.

The molecular weight was determined at pH 2.5 by the sedimentation equilibrium method with a protein concentration of 3.1 mg/ml, and by sedimentation and diffusion measurements at concentrations of 7.0 and 2.9 mg/ml. The results are in Table 1. The molecular weight is also given for unreduced gamma globulin at pH 2.5. The sedimentation equilibrium data were analyzed by the methods

suggested by Van Holde and Baldwin.[24] The slight curvature in the Z/r *vs.* $\int_a^r$ Zdr graph (Fig. 2), as well as the difference between $M_w$ and $M_z$ (Table 1), is probably a consequence of the small amount of 5 S material remaining in the sample. If the data are treated as resulting from a mixture of 90% of a monomer with molecular weight 75,000 and 10% dimer of weight 150,000, the values of $M_w$ and $M_z$ predicted are 82,100 and 88,100, in good agreement with the observed values. Since the sedimentation equilibrium method is extremely sensitive to small amounts of high molecular weight aggregates, it is important that the reduced material be passed through Sephadex G-200 before experiments of this kind are attempted. Preliminary experiments, in which this separation was not performed, gave erratic and high results.

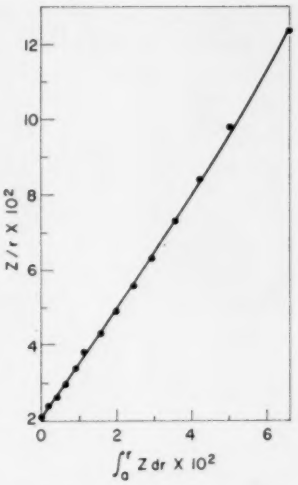


FIG. 2.—Sedimentation equilibrium of reduced gamma globulin in 0.1 *M* NaCl, pH 2.5. The data are graphed according to equation 35 of reference 24. Z is schlieren image deflection (cm). r is the distance from the center of rotation (cm), and a is the meniscus position.

The values obtained by measurements of sedimentation velocity and diffusion constant (Table 1) are in good agreement with the results of sedimentation equilibrium. The molecular weight obtained for unreduced gamma globulin at pH 2.5 falls within the range of values reported in the literature for rabbit gamma globulin at neutral pH.[21, 22, 25]

Reduction alone, without acidification, did not significantly affect the sedimentation coefficient; the $s_{20,w}$ value was 6.2 S in saline-borate buffer, pH 8.0, ionic strength 0.16. (It has previously been shown that treatment with 0.1 *M* MEA does not appreciably affect the sedimentation coefficient or specific viscosity.[12])

The results indicate that the 3.4 S component represents half molecules of gamma globulin. This is supported by the values obtained for the molecular weight and by the fact that it migrates as a single symmetrical peak in the ultracentrifuge. Additional support for this hypothesis is given by data (discussed below) indicating that the smaller subunits can apparently be formed without breaking the disulfide bonds linking "B chains" to the rest of the molecule.

*Effect of varying pH on the reduced gamma globulin molecule:* Portions of the

TABLE 1

MOLECULAR WEIGHTS AT PH 2.5*

| Gamma globulin preparation | Method | Protein concentration mg/ml | $s_{20,w}$ × $10^{13}$ | $D_{20,w}$ $cm^2/sec$ × $10^7$ | Molecular weight |
|---|---|---|---|---|---|
| Reduced† | Sedimentation equilibrium | 3.1 | | | $M_w$ = 81,800<br>$M_z$ = 85,500 |
| Reduced† | Sedimentation diffusion | 2.9 | 3.65 | 4.31 | 80,000 |
| Reduced† | " | 7.0 | 3.40 | 3.88 | 82,000 |
| Untreated | " | 8.1 | 4.55 | 2.75 | 155,000 |

* Solvent, 0.1 *M* NaCl containing sufficient HCl to lower the pH to 2.5.
† Details of the procedures used are in the text. The reduced samples were passed through Sephadex G-200 prior to determinations of molecular weight.

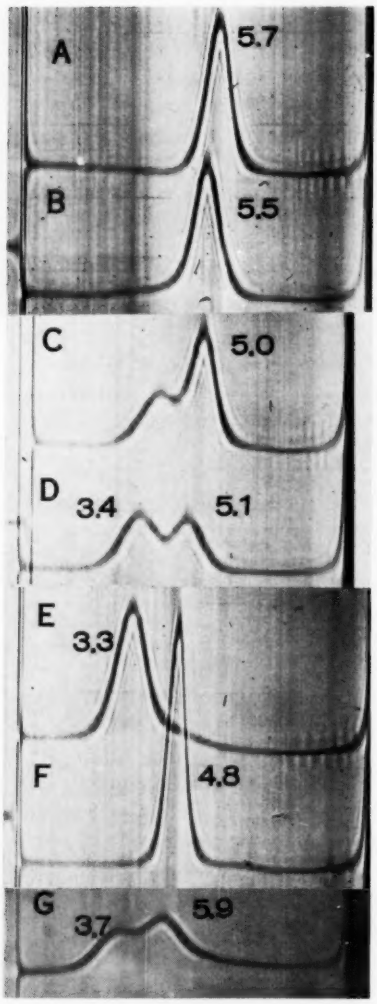


FIG. 3.—Photographs of schlieren patterns taken in a Spinco Model E ultracentrifuge after 80 min at 59,780 rpm (except *G*, 64 min). Sedimentation is from left to right. The numerals are $s_{20,w}$ values. *A*, unreduced gamma globulin, pH 3.3. *B*, *C*, *D*, and *E*: gamma globulin reduced with 0.1 *M* MEA and then adjusted to the following pH's: *B*, pH 3.3; *C*, pH 2.9; *D*, pH 2.7; *E*, pH 2.5. *F* is unreduced gamma globulin at pH 2.5. *G*, reduced sample adjusted to pH 7.0 after acidification to pH 2.5. Solvent, 0.1 *M* NaCl with sufficient HCl added to lower the pH to the desired value.

subunits which had not recombined.

reduced gamma globulin described in the preceding section were adjusted to various levels of pH after dialysis against 0.1 *M* NaCl. As already indicated, the sedimentation patterns of reduced and unreduced gamma globulins were similar at neutral pH. Figures 3*A* and 3*B* are photographs of the schlieren patterns of unreduced and reduced gamma globulin, respectively, at pH 3.3 (0.1 *M* NaCl + HCl). Again, little difference is noted in the patterns of S values (shown on the figure). Figure 3*F* indicates that lowering the pH from 3.3 to 2.5 decreased the $s_{20,w}$ value of the unreduced gamma globulin from 5.7 to 4.8 S. However, at this pH, the value for the reduced sample (3.3 S, Fig. 3*E*) is only 70% as great as that of the unreduced gamma globulin, as compared with 96% at pH 3.3. At intermediate pH's, 2.9 and 2.7, two distinct components are observed in the reduced sample (Figs. 3*C* and 3*D*). There is an increasing fraction in the form of the slower component as the pH is lowered; at pH 2.5 the transition is nearly complete (Fig. 3*E*). The small amount of faster-moving material visible in Figure 3*E* still remained at pH 2.0 (photograph not shown). These results indicate that the transition from whole to half molecules occurs between pH 3.3 and 2.5. Unreduced gamma globulin showed only a single component at pH's 2.9 and 2.7; $s_{20,w}$ = 5.0 and 4.9 S, respectively.

*Recombination of subunits at neutral pH:* The 3.4 S subunits formed by reduction and acidification appear to recombine spontaneously at neutral pH. Figure 3*G* shows the sedimentation pattern in 0.1 *M* NaCl after neutralization to pH 7. The $s_{20,w}$ value of the faster of the two major components, 5.9 S, agrees closely with the value for the same untreated gamma globulin (6.2 S); that of the slower component was approximately 3.7 S. This result suggests that the half molecules recombine to a large extent at neutral pH and that the slower component represents In a number of similar experiments 2 compo-

nents with similar S values were always obtained; however, the ratio of the amount of the faster to that of the slower component was usually greater than in Figure 3. In each experiment 10–20% of more rapidly migrating protein, presumably consisting of aggregates, was also present.

While the process is clearly reversible, the equilibrium cannot be rapidly attained, since two maxima are observed in the schlieren pattern at pH's 2.7 and 2.9. The theory of Gilbert[26] predicts that rapidly equilibrating monomer-dimer mixtures will exhibit only one maximum in the concentration gradient, moving at an intermediate velocity.

*Effect of reduction and acidification on the activity of antihapten antibody:* Tests of specific precipitability on recombined antibody were not reproducible. One recombined preparation of antiovalbumin gamma globulin formed a small amount of specific precipitate with antigen. Another specifically inhibited the homologous precipitin reaction (of untreated antibody with antigen). In view of the presence of some aggregates of high molecular weight and the possible presence of univalent molecules in the recombined preparations, interpretation of precipitin data is difficult. Since the method of equilibrium dialysis permits measurement of combining activity without the formation of specific precipitates, an antihapten antibody was investigated.

Forty-nine mg of anti-*p*-azobenzoate gamma globulin (3% precipitable by an ovalbumin-*p*-azobenzoate test antigen) was reduced with 0.1 *M* MEA as described above. Excess iodoacetate (0.2 *M*), rather than CMB, was used to react with free —SH groups, because CMB would be expected to combine specifically with the antibenzoate antibdy. After standing overnight in the cold, the mixture was dialyzed twice against 0.1 *M* NaCl, and the pH was lowered to 2.5. More than 85% of the protein migrated with $s_{20,w} = 3.3$ S, indicating a large amount of splitting into half molecules. After neutralization, two major components were observed with $s_{20,w}$ values of 3.6 and 5.7 S. Some aggregates were also present. Equilibrium dialysis was carried out with this preparation, with untreated antibenzoate gamma globulin, and with normal gamma globulin, each at a protein concentration of 16 mg/ml. The free concentration of hapten ($C^{14}$-*p*-nitrobenzoate) at equilibrium was $4.48 \times 10^{-6}$ *M*. The concentration of hapten bound by the treated and untreated antibodies and by normal gamma globulin were $2.46 \times 10^{-6}$, $2.91 \times 10^{-6}$, and $0.35 \times 10^{-6}$ *M*, respectively (averages of triplicate determinations). The results indicate that the specific binding capacity of the antibody is largely retained during reduction and acidification. (At a fixed free concentration of hapten, the concentration bound should be in direct proportion to the concentration of combining sites, if the average binding constant is not altered.)

*Separation of "B chains" from reduced samples:* To determine whether the disulfide bonds linking "B chains" to the remainder of the molecule are cleaved during reduction, we employed methods similar to those of Fleischman *et al.*,[7] except that Sephadex G-100 was used in place of G-75. Samples were dialyzed against two 4 liter portions of cold 1 *M* propionic acid, and the column was equilibrated with the same solvent. The ratio of column volume to the volume of sample exceeded 100 to 1. Samples of gamma globulin treated with 0.2 *M* mercaptoethanol and iodoacetate, as suggested by Porter,[8] yielded 2 peaks during chromatography. The second, which consists of "B chains,"[7] contained 20–25% of the protein. in close

agreement with the results of Fleischman *et al.* After treatment with 0.03 *M* MEA and CMB, by methods already described, 4.1 —SH groups per molecule were liberated. Only 6% of the protein was eluted from Sephadex G-100 as a second component; however, the same material gave 75% of a 3.3 S component in 0.1 *M* NaCl, pH 2.5. This indicates that the separation into half molecules does not require the release of free "B chains." A sample treated with 0.5 *M* MEA and CMB (11.2 —SH liberated) yielded 14% "B chains," and 85% of the 3.3 S component in 0.1 *M* NaCl, pH 2.5.

*Discussion.*—On the basis of analyses of carboxymethylcysteine content, after reduction of rabbit gamma globulin and alkylation with iodoacetate, Porter localized the interchain disulfide bonds and proposed that each "B chain" is held to an "A chain" by one disulfide bond and that the two "A chains" are linked by three disulfide bonds.[27]

Our results indicate that half molecules are formed from reduced gamma globulin at pH 2.5 and suggest that each consists of an "A chain" and a "B chain." This interpretation is compatible with the following facts: (*a*) the average molecular weight of the subunits formed at pH 2.5 closely approximates half the value for native gamma globulin. (*b*) The symmetry of the peak in the ultracentrifuge at pH 2.5 suggests that the subunits are similar in size. Other postulated combinations of "A" and "B chains" lead to molecular weights that are widely different from one another, or which disagree with the observed value. For example, a fairly symmetrical peak might result from the release of pairs of "B chains" and single "A chains," since the molecular weight of an "A chain" is about twice that of a "B chain," but the approximate average molecular weight would be only 50,000. (*c*) Only 2–3 disulfide bonds need be reduced for conversion to half molecules at low pH (0.1 *M* NaCl). This is consistent with Porter's model, if one postulates that the "B chains" remain attached to "A chains." (*d*) When an average of two disulfide bonds per molecule is cleaved, large amounts of half molecules are formed, but a low yield of free "B chains" is obtained by passage through Sephadex G-100 in 1 *M* propionic acid. This also indicates that the disulfide bonds linking the half molecules are more labile to reduction by MEA than those holding "B chains" to the remainder of the molecule.

When 0.5 *M* MEA is used for the reduction, a considerable proportion of "B chains" is separable in 1 *M* propionic acid, but remain attached to "A chains" in 0.1 *M* NaCl, pH 2.5, evidently through noncovalent bonds.

The transition to half molecules occurs between pH's 3.3 and 2.5 in 0.1 *M* salt (Fig. 3). The separation is probably attributable to electrostatic repulsion at low pH, which might disrupt hydrophobic bonds, or to the destruction of electrostatic bonds which require carboxylate ions. The apparent reformation of whole molecules at neutral pH (Fig. 3*G*) also supports the concept that noncovalent bonds are sufficiently strong to hold together the subunits. The large yield of 6 S protein that results after neutralization indicates that there is a strong tendency for these subunits to associate as pairs. The process is quite similar to the recently observed reversible dissociation of aldolase,[28, 29] the principal difference being the requirement in the case of rabbit gamma globulin for prior reduction of disulfide bonds.

Studies are in progress to attempt to define more precisely the conditions for dissociation and reformation, and to determine whether the half molecules are

univalent. The possibilities of reforming disulfide bonds after reassociation, by removal of CMB, and of hybridization of subunits from different antibody molecules are suggested.

* This work was supported by grants from the National Institutes of Health (A-3096 and E-3552) and the National Science Foundation

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# *DEPENDENCE OF FREQUENCY OF X-RAY-INDUCED CHROMOSOME ABERRATIONS ON DOSE RATE IN THE CHINESE HAMSTER**

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*Communicated by Bentley Glass, June 14, 1963*

In recent years several authors have investigated the production of chromosomal aberrations by ionizing radiations in various mammalian tissues.[1–4] In addition to these cytological investigations, several genetic studies have been performed on the effects of ionizing radiations in mammals.[5–7] Russell[7] has reported that in the mouse X-ray-induced mutation rates can be altered by changing the dose rate of a given dose of X rays, but no comparable study has been reported of a dose-rate effect on chromosomal aberrations in mammals. The experiments reported here investigate this effect.

In two studies of X-ray-induced chromosomal aberrations in the Chinese hamster, different breakage values were reported. Bender and Gooch[2] reported that in the bone marrow the dose curve for a 2-hr interval between treatment and fixation had a slope of 0.0051 breaks per cell per r, and the dose curve at a 6-hr interval between treatment and fixation had a slope of 0.0021 breaks per cell per r. In the corneal epithelium the slope of the dose curve for a 6-hr interval between treatment and fixation was 0.00352 breaks per cell per r.[4] In the latter experiments, no breakage value could be obtained when a shorter interval was used.

The results of the two studies are significantly different at a 5 per cent level of significance. Since in both experiments the spontaneous breakage rate was low (no spontaneous breaks were found in the bone marrow, and only 0.0085 breaks per cell in the corneal epithelium), the difference between the reported rates of induced breakage cannot be attributed to a greater instability of the unirradiated chromosomes in one system than in the other. It was thought that perhaps a dose-rate effect might account for the difference, since Bender and Gooch performed their irradiations at 60 r per min, and I performed mine at 600 r per min. The magnitude of the difference in these dose rates is very close to what Russell[7] has reported to have produced high and intermediate mutation rates.

That the frequency of radiation-induced chromatin breaks is proportional to dose was first demonstrated by Sax.[8] This general relationship holds, provided other factors such as oxygen tension, temperature, pH, etc., are kept constant.[9, 10]

On the other hand, many investigators[11–14] have shown that a second system, the rejoining system, plays a role in the types and frequencies of chromosomal aberrations observed. There appear to be two major components of the rejoining system, either of which, when lacking, has a profound effect on the observed frequency of two-hit aberrations. Production of adenosine triphosphate (ATP) must take place for the broken ends of chromosomes to rejoin, as experiments with metabolic inhibitors demonstrate.[11] Protein synthesis must also take place, for Wolff[15] found that when he added a known inhibitor of protein synthesis (chloramphenicol) to *Vicia faba* root tips during the interval between fractionated doses of X rays, he obtained the same frequency of two-hit aberrations as would be ex-

pected if the doses had not been fractionated. This observation indicates that the chromatin breaks remain open in the absence of protein synthesis.

The present experiments were performed primarily to determine whether dose rate has any effect on observable chromosomal damage and might account for the differences obtained in the breakage rates in the respective studies on bone marrow and on corneal epithelium. An experiment was also designed to determine whether the rejoining system is involved in any of the differences that might be observed.

*Materials and Methods.*—The cytological material used in all the experiments was the corneal epithelium of the Chinese hamster. All of the animals used were adult males. The hamsters were obtained from Dr. G. Yerganain of the Children's Cancer Research Foundation in Boston, Mass.

The animals were given whole-body X irradiation while clamped in a stationary position, as shown in Figure 1. The X rays were generated by a General Electric X-ray unit (Maxitron 250), with 1 mm of added aluminum filtration. All dose rates were determined by a Victoreen dosimeter in air. The X rays given at 600 r per min were generated at 110 kv and 30 ma with a target distance of about 11 cm; those given at 60 r per min were generated at 110 kv and 30 ma with a target distance of about 60 cm; and those administered at 2 r per min were generated at 110 kv and 5 ma with a target distance of about 1.5 meters.

Total doses of 50 r and 100 r were administered at the three dose rates, and fixation was performed at 3-, 4-, 5-, 6-, and 12-hr intervals thereafter for the 50 r dose, and at 6- and 12-hr intervals for the 100 r dose. In addition, the 50 r dose was repeated at all three dose rates while the eyes were being treated with puromycin (6-dimethylamino-9-[3′-deoxy-3′-(*p*-methoxyl-L-phenyl-alanyl-amino)-β-D-ribofuranosyl] purine), a known inhibitor of protein synthesis,[16, 17] with fixation at 6 hr after irradiation. The 50 r dose was administered at each of the selected dose rates, and the eyes were treated with saline A as a control for the puromycin experiment. Fixation was again performed after a 6-hr interval.

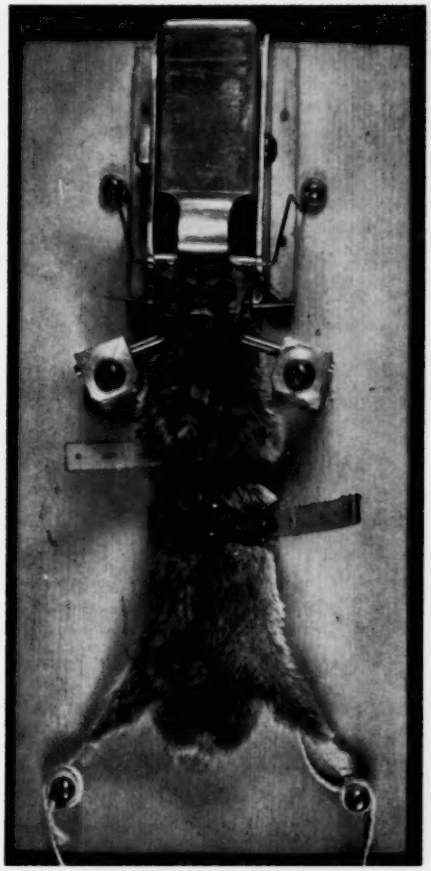

FIG. 1.—A hamster clamped in position in the apparatus used for all of the irradiations. The Lucite headpiece which was used to contain the solutions tested can be seen.

Puromycin was used in a concentration of 100 μg per ml of saline A. The animals which had the corneal epithelium treated with either puromycin or saline A had a Lucite apparatus fitted over the head (Fig. 1). The Lucite apparatus contained two holes bored at the hamster's interorbital distance. When fitted properly, each hole formed a well over an eye. The fur around the eyes and the Lucite apparatus were greased with Dow-Corning stopcock grease to produce a watertight seal. Each well was filled with the solution tested in such manner that the eyes were completely immersed in the solution. In the cases where the animals were irradiated at 2 r per min,

the wells were filled with the solution, and the irradiation was performed immediately. When the animals were irradiated at 60 r and 600 r per min, the wells were filled 24 and 25 min prior to the irradiation, respectively. In this way, all the treated eyes were exposed to the solution 25 min.

Immediately after irradiation, the wells were washed out several times with saline A. Controls were performed in which the puromycin or saline A solution was placed in the wells over the eyes for 25 min and then washed out, without any irradiation.

One and one-half hr before the animals were killed, they were given a 0.3 ml injection of a 1% colchicine solution under the skin of the abdomen. The animals were killed, and the eyes dissected out and placed in hypotonic saline solution (95% Hank's BSS without NaCl, 5% Hank's BSS with NaCl) at 37°C for 20 min. Cold, fresh 3:1 (ethyl alcohol:glacial acetic acid) was added, and the eyes were fixed at 0°C for 18 to 24 hr. The staining and mounting technique was the same as that previously described,[4] except that after Feulgen staining the cornea was washed in 45% acetic acid for 5 min to facilitate removal of the corneal epithelium.

Cells were preselected for scoring under low magnification (150×) and were scored at a magnification of 1,350×. Achromatic lesions in single chromatids were not scored as aberrations. Simple deletions, isochromatid deletions, and chromatid exchanges were scored. Any questionable aberrations seen in treated cells were scored as normal, whereas, in the control cells, all doubtful cases were scored as aberrations.

*Results.*—Figure 2 shows typical corneal epithelium metaphases prepared by

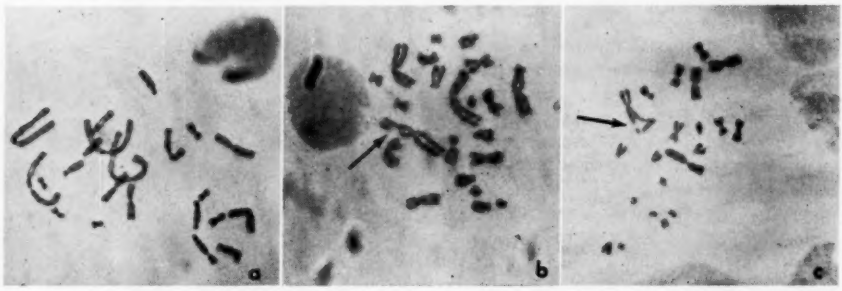


FIG. 2.—Typical metaphase preparations of the corneal epithelium of the Chinese hamster, prepared according to the method described in the text. (*a*) A normal, untreated diploid cell; (*b*) a cell containing a typical chromatid deletion; (*c*) a cell containing a chromatid exchange.

the method discussed in the text. In 336 normal, untreated cells scored at this time (the number includes 236 cells mentioned previously[4]), only 2 chromatid deletions have been observed. This is a spontaneous breakage rate of 0.006 breaks per cell. In 147 cells treated with puromycin at a concentration of 100 μg per ml and without irradiation, 2 chromatid deletions were observed, a breakage rate of 0.014 breaks per cell; while in the cells treated with saline A without irradiation, one break was observed in 140 cells—a breakage rate of 0.007 breaks per cell.

The complete data for all the experiments are summarized in Tables 1, 2, and 3. A mean value of breakage was calculated, for each dose rate used, from the 50 r and 100 r data obtained from fixation 6 hr after treatment (Table 2). At 2 r per min a breakage rate of $1.04 \times 10^{-3}$ breaks per cell per r was obtained, and for the 60 r and 600 r per min dose rates, breakage rates of $1.94 \times 10^{-3}$ breaks per cell per r and $2.95 \times 10^{-3}$ breaks per cell per r, respectively, were obtained (Table 2). The values at the 2 r per min and 600 r per min dose rates are significantly different at the 5 per cent level of significance (Fig. 3), and it can be assumed that the data

TABLE 1

FREQUENCY OF CHROMATID ABERRATIONS IN THE CORNEAL EPITHELIUM OF THE CHINESE HAMSTER EXPRESSED AS PER CENTS

| Dose | Dose rate | Cells scored (N) | Deletions | Isochromatids | Exchanges | Total* breaks |
|---|---|---|---|---|---|---|
| | | (6 hr after treatment) | | | | |
| | 2 r/min | 176 | 3.5 | 0.0 | 1.1 | 5.7 |
| 50 r | 60 r/min | 200 | 8.0 | 2.0 | 0.5 | 11.0 |
| | 600 r/min | 175 | 11.5 | 2.9 | 1.1 | 16.6 |
| | 2 r/min | 140 | 9.3 | 2.1 | 0.0 | 11.4 |
| 100 r | 60 r/min | 145 | 9.7 | 2.7 | 2.8 | 18.0 |
| | 600 r/min | 147 | 17.7 | 3.9 | 2.8 | 27.2 |
| | | (12 hr after treatment) | | | | |
| | 2 r/min | 40 | 2.5 | 0.0 | 0.0 | 2.5 |
| 50 r | 60 r/min | 143 | 3.5 | 0.0 | 0.0 | 3.5 |
| | 600 r/min | 200 | 3.0 | 0.5 | 1.5 | 6.5 |
| | 2 r/min† | — | — | — | — | — |
| 100 r | 60 r/min | 150 | 5.3 | 0.7 | 0.7 | 7.3 |
| | 600 r/min | 100 | 9.0 | 1.0 | 2.0 | 14.0 |
| | Control | 336 | 0.6 | 0.0 | 0.0 | 0.6 |

* Deletions plus isochromatids plus twice chromatid exchanges.
† Materials lost during staining procedure.

TABLE 2

FREQUENCY OF CHROMATID ABERRATIONS PER CELL PER R 6 HR AFTER TREATMENT

| Dose | Dose rate | Cells scored (N) | Deletions ($\times 10^{-3}$) | Isochromatids ($\times 10^{-3}$) | Exchanges ($\times 10^{-3}$) | Total* breaks ($\times 10^{-3}$) |
|---|---|---|---|---|---|---|
| 50 r | 2 r/min | 176 | 0.70 | 0.00 | 0.22 | 1.01 |
| | 60 r/min | 200 | 1.60 | 0.40 | 0.10 | 2.08 |
| | 600 r/min | 175 | 2.30 | 0.58 | 0.22 | 3.20 |
| 100 r | 2 r/min | 140 | 0.93 | 0.21 | 0.00 | 1.08 |
| | 60 r/min | 145 | 0.97 | 0.27 | 0.28 | 1.74 |
| | 600 r/min | 147 | 1.76 | 0.39 | 0.28 | 2.66 |
| Mean | 2 r/min | 316 | 0.80 | 0.09 | 0.12 | 1.04 |
| | 60 r/min | 345 | 1.33 | 0.34 | 0.18 | 1.94 |
| | 600 r/min | 322 | 2.05 | 0.49 | 0.25 | 2.95 |

* Deletions plus isochromatids plus twice chromatid exchanges.

TABLE 3

FREQUENCY OF CHROMATID ABERRATIONS IN CORNEAL EPITHELIUM CELLS TREATED WITH PUROMYCIN OR SALINE A PLUS X-IRRADIATION, 6 HR AFTER TREATMENT

| Dose | Dose rate | Cells scored (N) | % Deletions | % Isochromatids | % Exchanges | % Total* breaks | Breaks/cell/r |
|---|---|---|---|---|---|---|---|
| | | | (Puromycin plus X-irradiation) | | | | |
| 50 r | 2 r/min | 100 | 14 | 1.0 | 2.0 | 19 | $3.52 \times 10^{-3}$ |
| | 60 r/min | 100 | 12 | 1.0 | 2.0 | 17 | $3.12 \times 10^{-3}$ |
| | 600 r/min | 100 | 12 | 2.0 | 2.0 | 18 | $3.32 \times 10^{-3}$ |
| | Control | 147 | 1.4 | 0.0 | 0.0 | 1.4 | |
| | | | (Saline A plus X-irradiation) | | | | |
| 50 r | 2 r/min | 113 | 4.4 | 0.9 | 0.0 | 5.3 | $0.92 \times 10^{-3}$ |
| | 60 r/min | 100 | 8.0 | 1.0 | 0.0 | 9.0 | $1.66 \times 10^{-3}$ |
| | 600 r/min | 100 | 13.0 | 2.0 | 1.0 | 17.0 | $3.26 \times 10^{-3}$ |
| | Control | 140 | 0.7 | 0.0 | 0.0 | 0.7 | |

* Deletions plus isochromatids plus twice chromatid exchanges.

at the 60 r per min dose rate would be significantly different from the other two, had a larger sample been taken. The reduction in the total breakage as the dose rate is decreased is also evident in the data obtained from a 12-hr fixation time (Table 1).

The breakage rate obtained in these experiments at the 600 r per min dose rate is in good agreement with the value of $3.52 \times 10^{-3}$ breaks per cell per r obtained by the author in earlier experiments.[4] Likewise, the value obtained here at the

60 r per min dose rate is in very good agreement with the value of $2.1 \times 10^{-3}$ breaks per cell per r obtained by Bender and Gooch[2] at the 6-hr interval between treatment and fixation in the bone marrow of the Chinese hamster with a dose rate of 60 r per min. These close agreements indicate that what was previously regarded as a discrepancy between the results of different workers, or as a specific effect of the tissue treated, may be attributable to differences of dose-rate effects.

In order to determine whether the degree of mitotic delay was significantly different at the three dose rates, and whether, consequently, the cells observed at a 6-hr fixation time for each dose rate had been differentially affected because they were in different stages of mitosis at the time of irradiation, an additional series of animals was irradiated with 50 r at the 2 r and 60 r per min dose rates; and fixation was performed at 3-, 4-, and 5-hr intervals thereafter. In all but one animal of both the original and repeated series, metaphase figures were absent for intervals shorter than 6 hr. In one animal at the 5-hr fixation time, irradiated at 60 r per min, 89 metaphases were scored and 7 single breaks observed. The latter case was interpreted as indicating the beginning of the burst of mitosis following delay and as being composed of those cells which were least affected or which underwent the least amount of regression, whereas the cells fixed at the 6-hr interval in the first series probably represented the peak of the mitotic burst and were therefore a more heterogeneous sample of the cell population. The fact that the breakage value is lower at the 5-hr interval than at the 6-hr interval supports this view.

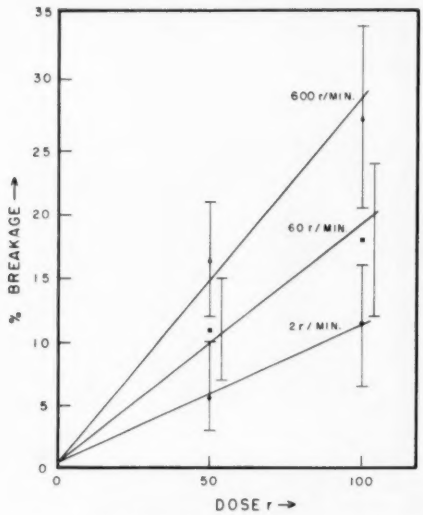


FIG. 3.—Relationship of observed breakage to dose at the three dose rates studied. The measures of significance indicated for each point are the 95% probability limits, calculated according to the method of Stevens.[20]

In the series of experiments in which the corneal epithelium was treated with puromycin and also irradiated, the breakage values were the same at all three dose rates (Table 3) and equivalent to the highest dose rate in the absence of puromycin. In the experiments where the corneal epithelium was treated with saline A and irradiation was performed, the breakage values were in close agreement with those obtained with no treatment other than irradiation. Evidently, the effect of puromycin is to abolish the protection derived from a low dose rate.

Figure 3 shows the relationship of observed breakage to dose at each of the three dose rates used. In Figure 4, the relationship of breakage to dose rate is plotted.

*Discussion.*—The observation that a lower frequency of breakage is observed as the dose rate decreases might be attributed to any one of several causes. (1) Cells may have been in different stages of mitosis at the time of irradiation. Since they were uniformly scored at a 6-hr interval after irradiation, there might be

differences in mitotic delay at higher and lower dose rates. (2) A differential oxygen effect might exist. One way for this to occur would be by differences in adrenaline titer in the tissues of animals irradiated at different dose rates. Animals irradiated at the low dose rates, because they had to be immobilized in the apparatus used for the treatments for a long period of time, might become afraid and struggle to get free. They might thereby increase the adrenaline titer in their tissues, lower the oxygen tension because of heightened cellular respiration, and decrease the effect of irradiation. (3) There might be a lower frequency of induced breakage at the lower dose rates. (4) The rejoining system might be differentially affected at different dose rates. If so, one would expect a differential degree of rejoining at the three dose rates.

Bender and Gooch[4] have demonstrated that observable damage in the bone marrow of the Chinese hamster decreases as the interval between treatment and fixation increases. Their data at 2-hr and 4-hr intervals show a higher breakage rate at 60 r per min than the present data show at the 6-hr interval irradiated at 600 r per min. However, this difference may indicate only that they were observing cells which were in metaphase or entering it at the time of irradiation. The lack of chromatid exchanges in their 2- and 4-hr data supports such a view. Bender and Gooch's 6-hr data are the first to show any chromatid exchanges, a fact indicating that the cells scored at this time were probably in early to mid-prophase when irradiated. Perhaps the lack of metaphases at the early fixation times in the corneal epithelium can be explained by the relative delay in access of colchicine to that tissue after injection. The cornea is an avascular tissue; the bone marrow has a direct blood supply. Hence, colchicine must reach the corneal epithelium by diffusion, a process requiring more time than is needed for colchicine to reach a tissue through the vascular system, as in the case of the bone marrow. A prolonged delay between injection and the access of the colchicine to the tissue might allow cells, in or entering metaphase at the time of irradiation, to complete metaphase before mitosis was stopped. The lack of metaphase figures after early fixation times makes it reasonable to believe that the observed differences in breakage at the three dose rates are not due to a variation in the degree of mitotic lag. It would follow that at the 6-hr interval, cells are observed which at all dose rates were in the same stage of mitosis at the time of irradiation.

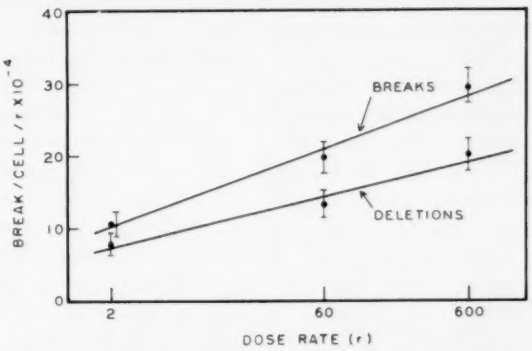


FIG. 4.—The relationship of observed breakage to dose rate. The measures of significance at each point are the 95% probability limits calculated according to the method of Stevens.[20]

The experiments in which three hamsters were treated with saline A prior to and during irradiation shows that the length of time the animals were clamped in the apparatus and consequently the amount of adrenaline introduced into the

blood stream as a result of their struggles was not the cause of the observed variations. In this experiment the observed breakage rates were the same whether the animals were in the apparatus for a long or short period of time. Since it is generally accepted that initial breakage is only dose-dependent and does not vary with dose rate, the only remaining explanation of the differences in the data is that of a differential effect of the dose rate on the chromosome rejoining system.

Newcombe[18] has suggested that the amount of rejoining decreases as both dose and dose rate increase. He found that all types of aberrations (one- and two-hit) varied with dose rate and postulated that this variation is a function of the rejoining system. Although his data are at variance with most of the other existing data in respect to the effect of dose rate, his theories of the existence of a rejoining system have been proved to be correct. A possible explanation of the variation in the number of deletions he observed under different conditions could be that the frequency of all types of chromosomal aberrations is dependent on three factors: (1) the frequency of initial breakage, which is dose-dependent; (2) damage done to the rejoining system, which would affect the length of time the breaks remain open; and (3) the proximity of the broken ends, which would determine the probability of interaction and restitution of the breaks.[10] Three things can occur when multiple chromosomal breaks are induced in a cell: (1) the broken ends can restitute normally, resulting in no observable cytological aberration; (2) they can remain open and give rise to one-hit aberrations; or (3) interaction of the broken ends can occur to produce a two-hit aberration. Restitution and interaction both are dependent on the rejoining system, since healing or fusing of broken ends is necessary. However, at low doses, where the probability of two breaks occurring in a single cell is low, the frequency of two-hit aberrations will be low, regardless of what factors serve to keep the breaks open. It is consequently not likely that damage done to the rejoining system would be manifested in a noticeable change in the frequency of two-hit aberrations at low doses. Such variation probably would be confined to the frequency of one-hit aberrations, as appears to be the case in the present experiments.

In the present experiments, when puromycin was used to treat the cells during the irradiation, the same breakage rate, at the level of the high dose rate in the controls, was obtained at the three dose rates employed. Since puromycin inhibits protein synthesis and since protein synthesis is necessary for rejoining, equivalence of the effect at all three dose rates when puromycin was present probably results from equal inhibition of the rejoining system. When puromycin was not used, however, the frequency of aberrations differed at the three dose rates. It seems that a differential effect is exerted on the rejoining system by the three dose rates.

The work of Wolff and Luippold[19] showed that there are two types of breaks with respect to the length of time they remain open. On this basis, it seems reasonable to postulate that the observed effects of dose rate result from the amount of damage done to the rejoining system per unit of time at different dose rates. If we suppose that a total dose of Y roentgens causes a fixed amount of damage, X, to the rejoining system regardless of the dose rate, then those treatments in which the total dose is administered in a very short time, such as 5 or 10 sec, will produce X damage before any appreciable rejoining can occur. However, if the length of time over which the damage is produced is much greater, e.g., 50–100 sec or 25–50 min, as

in these experiments, some rejoining may occur before the total amount of X damage is done to the rejoining system. At the higher dose rates the broken ends might become separated before the rejoining system has recovered and is functioning normally. That would further reduce the probability of rejoining.

As in the case of mutations,[7] the observed frequency of chromosomal aberrations is dependent not only on dose but also on dose rate. The present analysis leads to the conclusion that this effect is probably mediated through the rejoining system.

*Summary.*—When the corneal epithelium of the Chinese hamster is irradiated with X rays at three different dose rates, the observed frequency of chromatid aberrations is different at each of the dose rates employed. This difference is manifested in the frequency of chromatid deletions as well as chromatid exchanges. When the corneal epithelium is treated with puromycin at the three dose rates, the same frequency of aberrations is observed at all three dose rates. This frequency is the same as that observed at the highest dose rate without treatment with puromycin. Puromycin inhibits protein synthesis, and protein synthesis is known to be necessary for the rejoining of chromosomal breaks. Therefore, it appears that the effect of dose rate is a differential effect on the rejoining system.

The author gratefully acknowledges the assistance of Dr. Bentley Glass and Dr. T. Merz. The puromycin used in the experiments was obtained from Dr. M. B. Yarmolinsky.

* This work was supported by the Atomic Energy Commission and was done while the author was a National Institutes of Health predoctoral fellow.

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# *POLARIZATION OF CELL LOCOMOTION IN VITRO**

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*Communicated June 28, 1963*

Cell locomotion is commonly referred to under such descriptive terms as chemotaxis, galvanotaxis, phototaxis, or thigmotaxis, without identification of the underlying mechanisms. This paper contributes one analytical result.[1]

The oriented displacement of a cell relative to its environment is due either to passive convection (e.g., by liquid flow or electrophoresis) or to active locomotion. The latter is biologically more relevant but analytically less well understood. As summarized in an earlier paper[1a] it has three distinct and separable aspects relating, respectively, to the motive force, the pathway, and the direction along the pathway. The motive force is provided by metabolic energy, which enables the cell to expand its surface. Contact with a planar or fibrous structure restricts surface expansion to disk or spindle-shape, hence functions as pathway or track ("contact guidance," Weiss[2]). Applied to a linear track, a bipolar spindle cell is still free to move in two opposite directions. In a statistically isotropic environment, a bipolar cell with statistically equivalent poles would therefore remain stationary, shuttling about dead center in accordance with random variations at its two motile tips. To yield directional displacement, this random variation must be overlaid by a persistent polar asymmetry letting one pole advance more actively than the other. With Child,[3] one would look for the source of this asymmetry in the cellular environment. The egg of the seaweed, Fucus, can be polarized by external gradients of electric potential, pH, or light.[4] If environmental asymmetries were to have similarly polarizing effects on cell locomotion, these could serve as clues to the mechanism underlying the particular "taxis." We therefore undertook to test the locomotor behavior of single cells in culture subject to pH gradients and to electric potential gradients, reasoning as follows:

The motility of the free margin of a cell varies locally with the state of its surface, which in turn is a function of the metabolic and physicochemical conditions in the particular cytoplasmic sector. Recent experiments in this laboratory[5] had revealed that the physicochemical state of an isolated cultured cell changes drastically if the pH of the medium is raised or lowered from its normal level (at 7.4). Above pH 8, the cell contracts vigorously so that a spread-out cell withdraws its pseudopodia and may even detach itself from the substratum; whereas near pH 6 and below, the cell becomes gradually immobilized, as all motion in the interior, signalled by granular or mitotic movements, and of the surface, signalled by the waving of the free border, comes to a standstill in whatever the momentary configuration of the cell may have been. Both of these pH effects are fully reversible, at least within a few hours, upon return to normal medium. The uniformity of the medium in these experiments has left it undecided whether the cell reacts to the pH change as a whole or locally. Could a limited sector of the circumference of a cell be made to contract or be paralyzed locally if that part of its surface were exposed to a pH value higher or lower than the rest?

*pH Gradients.—Technique:* We used cells taken from a stock culture of human

conjunctiva or from freshly dissociated heart or liver of chick embryos, separated according to the standard methods of this laboratory.[5, 6]

In preliminary experiments, chambers as illustrated in Figure 1 were used. A suspension of cells in Eagle's medium with 10% beef embryo serum added ("regular medium") was placed in the narrow gap (G) between the glass coverslip (C) and the plastic block (P), where it was held by surface tension. One edge of the liquid film was exposed to air saturated with water vapor, and the other to saturated $CO_2$. Since the pH of the bicarbonate-buffered medium reflects the local concentration of $CO_2$ dissolved in it, a pH gradient along the film was thus established, the extremes being estimated to be about 9 at the aerated end, and 6 at the $CO_2$ end.

Within a few minutes, the cells near the alkaline end rounded up and tended to detach from the glass, whereas those near the acid end became immobile and frozen in appearance. Cells in the zone of intermediate pH moved, but with no preferred direction, as the pH differentials across individual cells were evidently too small. For the smallest practicable shelf (3 mm wide), the average gradient is one pH unit per millimeter, hence only 0.05 units over the 50$\mu$ diameter of a spread conjunctival cell; some unavoidable turbulence reduced the pH differential still further.

To produce a steeper gradient a radial system was used. The suspension of cells was placed in a narrow gap between two circular coverslips, the upper one containing a central hole (about 200$\mu$ in diameter), near which the medium was exposed to a $CO_2$ atmosphere while the circumference was exposed to air. The pH gradient, although steeper near the hole than in the previous case, was still insufficient to affect cellular locomotion demonstrably.

Success was finally achieved by exposing opposite ends of a single cell to streams of media of different pH. The streams issued from glass capillary pipettes, mostly about 20$\mu$ in diameter and 50$\mu$ apart. For a pH difference of 1.2 units between the streams the gradient is therefore 24 pH units per mm, 24 times greater than in the method described above. The actual pH gradient over the cell diameter may be somewhat less because of the possible presence of a thin layer of unstirred medium at the cell surface or the admixture of some of the surrounding medium to the streams by their turbulence. The two pipettes were cemented so as to be moved as a unit. In one series, they were moved by means of a de Fonbrune micromanipulator. Each pipette was connected by flexible polyethylene tubing to a reservoir bottle, the height of which could be altered to adjust the rate of flow.

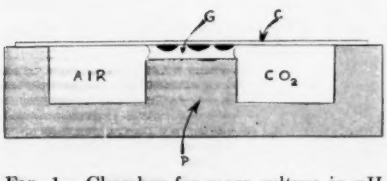



FIG. 1.—Chamber for mass culture in pH gradient.

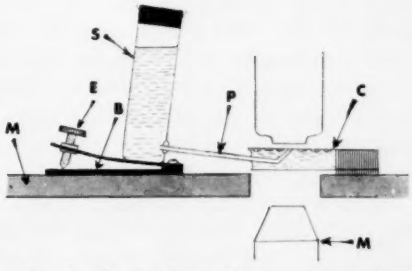



FIG. 2.—Device for setting up pH gradients across single cells in culture. Only one of the two pipettes and supply bottles is shown.

Since polyethylene is permeable to carbon dioxide, pH changes occurred along the slowly moving columns of medium. To prevent this a larger polyethylene sleeve was placed around each supply tube, and a $CO_2$-air mixture with which the medium was in equilibrium (see below) was passed through the sleeve.

In another series of experiments (Fig. 2), the two small reservoir bottles (S) and glass pipettes (P) were mounted as a unit on a brass base (B) that could be slid manually on a thin layer of heavy grease on the microscope stage (M). The pipettes could thus be accurately positioned in the microscope field and in relation to the cells. Vertical adjustment was achieved by means of the screw (E) which tilted the brass leaf supporting the bottles.

The cell chamber, approximately 4 mm in height, consisted of stainless steel or Lucite. A rectangular opening approximately 2 cm square was cut out, and glass coverslips (C) were waxed above and below. A suspension of cells in regular medium was held by surface tension in the shallow cavity, the cells spreading on one of the glass surfaces. The pipettes entered the chamber through one side, which was open to the atmosphere, although sometimes the exposed surface was covered with microscope oil (Cargille's Type A) to prevent evaporation and pH changes.

Flow rate through the pipettes was estimated from the advance of dye along the wider part of the capillary tube. For a tip lumen of $20\mu$, the nozzle speed was estimated to be 3 $cm/sec^{-1}$ for a pressure corresponding to a head of 2.7 cm of water. As the stream fanned out after leaving a pipette, its velocity of course fell off approximately as the square of its diameter. The volume flow through each pipette was about 0.5 $mm^3$ per minute and was made visible by the movement of particles of mycostatin added to suppress fungal growth.

In most experiments, one pipette carried a control stream of "neutral" medium (equilibrated with a 5% $CO_2$ atmosphere; pH about 7.4). The other stream was arranged to be either more acid (about pH 6.2) or more alkaline (about pH 8.6) by appropriate adjustments of the $CO_2$ content.

In a number of experiments the exposed cells were photographed serially on Polaroid film with phase contrast optics, usually at 320 × magnification.

*Results.*—Typical examples of the effect of exposing an isolated cell to an alkaline-neutral pair of streams are shown in Figures 3 and 4. A conjunctival cell usually begins to round up and to become detached from the glass on the alkaline side within a few minutes, while the other side of the cell, flushed by the neutral stream, remains unaffected. As the detachment on the alkaline side releases the strain on the erstwhile stretched cell, the cell body is pulled into the neutral region. Moreover, if the cell margin on the neutral side is in active advance, the cell will continue to progress in this direction for lack of a constraining rear attachment.

If at the time the pH gradient was actuated, a cell was moving toward the now alkaline side, its progress in that direction would be halted. It would retract from this side and eventually would either reverse its direction or move off along another path clear of the alkaline stream.

Figure 5 exemplifies the effect of alkaline-neutral streams on cell groups. The cells (embryonic chick liver) near the alkaline stream have rounded up, some of them having been actually flushed off, whereas those near the neutral stream are moving actively away from the alkaline area.

In one experiment, the alkaline stream was produced not by reducing the amount

of dissolved $CO_2$ but by increasing the amount of sodium bicarbonate (and commensurately reducing the sodium chloride concentration for isotonicity). This medium had a pH of 8.3 in equilibrium with 5% $CO_2$, whereas the pH of regular medium in this atmosphere is 7.4. A chick embryo heart cell exposed to these streams rounded up on the alkaline side. This result corroborates Taylor's[5] demonstration of the hydrogen ion concentration (pH), rather than the concentration of dissolved $CO_2$ as such, being the relevant factor in the described phenomena.

Turning now to experiments with acid-neutral pairs of streams, typical results are shown in Figures 6, 7, and 8 for human conjunctival cells, and in Figure 9 for chick embryo liver cells. As is clearly seen, cell processes in the acid stream become congealed and immobilized, while on the neutral side the cell continues to be mobile and accordingly moves off in that direction. Since the paralyzed end still remains attached to the glass, as the main body of the cell moves away, it is often drawn out into long narrow extensions before its tip finally becomes detached.

In a few experiments, in which cells were placed in an acid-alkaline pair of streams, they rounded up on the alkaline side, then pulled across to the acid stream, and there became totally immobilized.

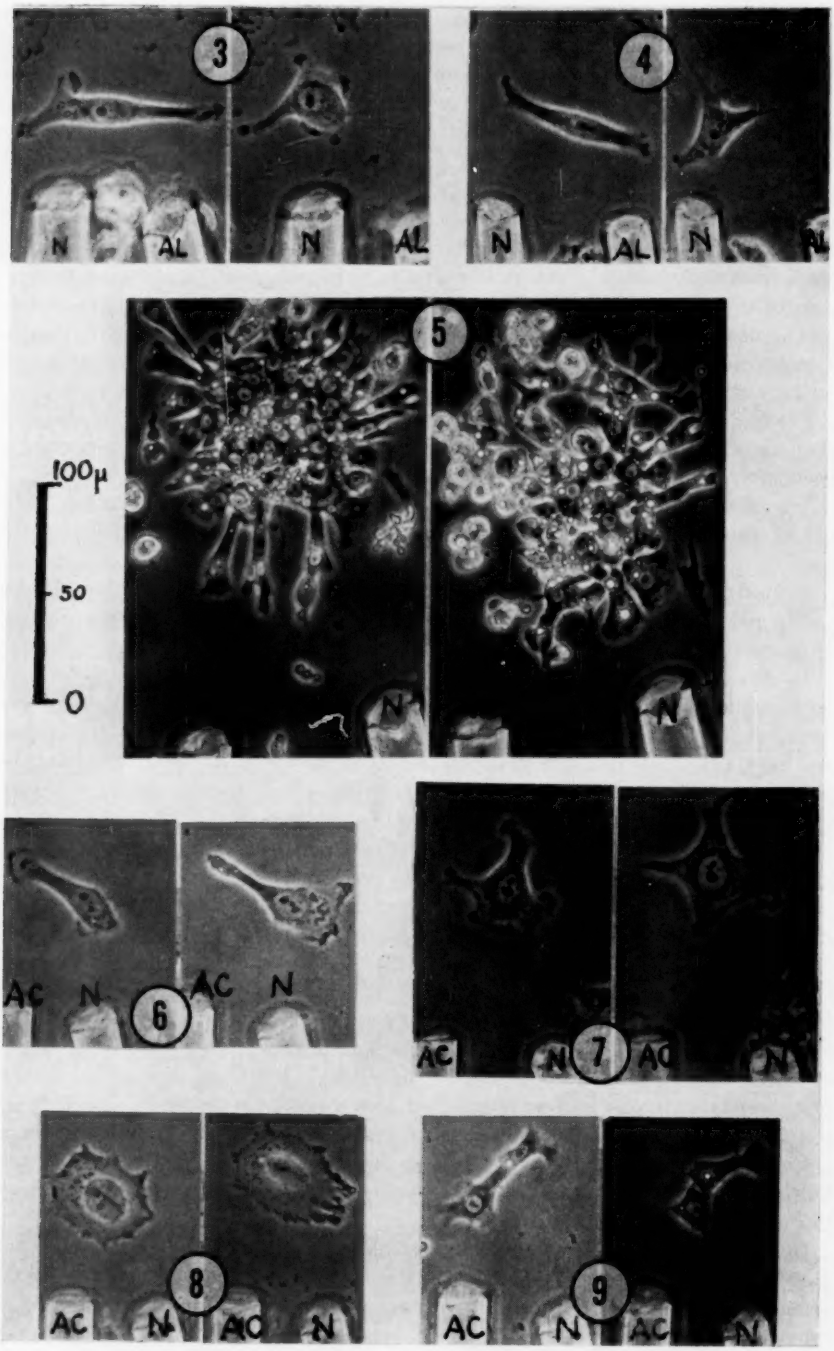
The effect of pH gradients on the movement of amoebae was also studied. The pH of spring water was adjusted to values within the range 4.5 and 9.5 using HCl and NaOH. When subjected to acid-alkaline streams within this range, amoebae continued to move randomly, their internal streaming being not perceptibly affected by the pH of the surrounding medium. At pH 3.5, amoebae stopped moving and appeared dead.

*Electric Potential Gradients.*—Effects of electric currents on living organisms have been studied for more than 100 years (see Lund *et al.*[7]). Most of these studies have failed to distinguish critically between the effects of the currents themselves and chemical effects due to asymmetrical concentrations of ions resulting from the passage of current. With due regard to the distinction, slime molds were shown to be negatively "galvanotactic."[8] Nerve fiber growth *in vitro* showed deflection away from the anodal side.[9] These results suggest local cell paralysis opposite the anode as the directive mechanism.

To test this assumption, we subjected cells spread on glass surfaces in culture medium to an applied current. Care was taken to prevent electrolytic products from reaching the cultures, either by connecting electrodes and cell chamber through long tubes filled with a 2% agar gel made up with Earle's solution and replaced regularly, or by the method shown in Figure 10. Cells were confined to a channel of dimensions $1 \times 1 \times 20$ mm in a Lucite block, covered with coverslips, and plugged at either end with the Earle's solution agar (A). A stream of Earle's solution first flowed slowly past each plug and then past a platinum electrode, thus keeping the electrolytic products of the latter downstream and away from the cell chamber.

Current was supplied from dry cells, its magnitude being measured by the potential drop across a known resistance in series with the chamber, using a Keithley model 603 electrometer.

Current densities in the medium greater than 8 mA $mm^{-2}$ were lethal to spread human conjunctival cells. Cells detached and rounded up quickly, and their membranes were found to be ruptured. Since the conductivity of regular medium is approximately $1.8 \times 10^{-2}$ mho $cm^{-1}$, it can be seen that a spread cell 50$\mu$ in extent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is killed if the potential difference between its ends exceeds about 0.2 volts. No attempt was made to determine the current actually passing through the cell itself.

In a large series of experiments performed with lower current densities, the cells (human conjunctiva, embryonic chick heart or liver) appeared to behave normally, moving freely on the glass surface. However, no preferential direction of motion was observed, cells appearing to move as frequently toward the anode as the cathode.

In some experiments the conductivity of the external medium was reduced by replacing part or all of the salts in regular medium with an osmotically equivalent amount of glucose. Except for a lowering of the lethal current density, due to a greater fraction of current passing through the cells, the results were the same as before.

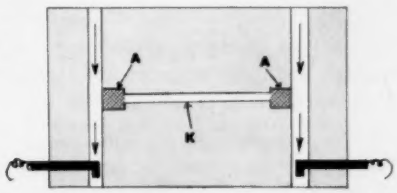


FIG. 10.—Chamber for culture in electric potential gradients.

Under the conditions of these experiments, therefore, isolated cells in tissue culture show no galvanotactic response.

*Conclusions.*—The experiments have established the following facts and conclusions:

(1) The cellular response to environmental pH is a local one, each sector of the cell behaving in accordance with the pH of its immediate vicinity.

(2) Locally applied, alkalization produces local surface contractions, while locally applied acidification entails local gelation.

(3) Either effect paralyzes the affected cell sector for locomotor purposes, even though by different means, thus leaving the cell to one-sided traction by its unaffected side.

(4) A maintained pH gradient thus can be instrumental in polarizing cell locomotion, with the following qualifications. The required steepness of 24 units $mm^{-1}$, effective in our experiments, is unlikely to be a natural occurrence in open tissue systems richly bathed with fluid. However, it is by no means beyond the range of plausible differentials in the microenvironment of closely clustered cells. The local release of $CO_2$ or lactic acid by a focal cell or cell group in high metabolic activity would be sufficient to establish a pH gradient of requisite magnitude between it and its less active neighbors, thus setting up a "field" of dynamic sequelae of inhibitions, centrifugal movements, and so forth.[3, 10]

(5) The mutual paralysis between contacting tips of mesenchyme cells ("contact inhibition"[11]), often associated with retraction and detachment of colliding processes,[12] bears many features suggestive of a local pH change at the contact point resulting from chemical interaction. The reported mutual "repulsion" between pigment cells in capillaries[13] can similarly be explained by a reciprocal paralysis of the cell sides facing each other as $CO_2$ accumulates there and lowers the pH.

←

FIGS. 3–9.—Changes in configuration and locomotion of cells between neutral and alkaline (Figs. 3–5) and neutral and acid (Figs. 6–9) streams. Each pair of illustrations composing these figures represents the same cell at the beginning of an experiment (left) and some time later (right). The intervals for Figs. 3–9 were 20, 40, 100, 60, 25, 110, and 100 min, respectively.

(6) The role of pH gradients in the cell shifts of early wound healing should be examined in this light. From our results, one could anticipate that in internal lesions (e.g., gastric ulcers), any deviation from an optimal pH in either the acid or the alkaline direction would impair wound coverage by cell migration. Similar considerations may apply to the positively or negatively "chemotactic" reaction of cells toward infectious foci or tumors.

(7) The external asymmetries determining the direction of cellular advance along a given track have, in the cases tested thus far, turned out to be attributable to rear inhibition, rather than to front stimulation, of cells endowed with endogenous motility. In conjunction with earlier results,[14] this strengthens the conclusion that, contrary to physical "attractions," any observed acceleration of cell advance, for a given metabolic state, is due to the reduction of physical restraints rather than to active physical promotion.

* These investigations were supported in part by grants from the American Cancer Society, the National Science Foundation, and the National Cancer Institute (National Institutes of Health of the U.S. Public Health Service).

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## *POLYRIBOSOMES AND PROTEIN SYNTHESIS DURING RETICULOCYTE MATURATION IN VITRO**

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*Communicated by David Shemin, June 19, 1963*

Erythroid cells provide a particularly suitable model for studying the factors regulating mammalian cell maturation. A variety of structural and biochemical changes are known to occur as erythroid cells mature, such as the onset of hemoglobin formation, the loss of the ability to synthesize DNA and to divide, the extrusion of the nucleus, and, eventually, as the reticulocyte matures to the erythrocyte, the

complete cessation of protein synthesis.[1-4] In reticulocytes, which synthesize protein, there is no detectable synthesis of ribosomal or messenger RNA.[5] In these cells, protein synthesis proceeds primarily on polyribosome structures which are clusters of 2 or more 78S ribosomes.[5-7] The present experiments are designed to determine if the decrease in protein formation which occurs as reticulocytes mature reflects a change in the clustered ribosomes. It has been found that 80–90 per cent of the ribosomes in a population of reticulocytes at the outset of maturation are arranged in clusters. As these cells mature *in vitro* to erythrocytes, the decrease in ability to synthesize protein is associated with a loss in the content of polyribosomes.

*Methods.—Conditions for the maturation of reticulocytes in vitro:* Reticulocytes were prepared from phenylhydrazine-treated rabbits according to the procedures of Borsook *et al.*[8] The cells were recovered from the whole blood by centrifugation and suspended in 2.0 volumes of a medium of the following composition: a mixture of L-amino acids,[9] 0.12 *M* NaCl, $4.75 \times 10^{-3}$ *M* KCl, $1.18 \times 10^{-3}$ *M* $MgSO_4$, $1.18 \times 10^{-3}$ *M* $KH_2PO_4$, $2.5 \times 10^{-2}$ *M* $NaHCO_3$, $2.4 \times 10^{-5}$ *M* $FeCl_2$, $3.6 \times 10^{-3}$ *M* glucose, and $6 \times 10^{-4}$ *M* inosine, plus 2.0 volumes of plasma and 100,000 units of penicillin and 0.25 gm streptomycin per 100 ml of mixture. Both plasma and cells were obtained from the same rabbit. This suspension, generally in a final volume of 40 ml, was placed in a siliconized, water-jacketed incubation flask fitted with a cap, through which passed a pH electrode, stirring rod, burette tip, and gas inflow and outflow tubes. The pH of the incubation mixture was maintained constant at 7.37 by means of a pH-Stat (Radiometer Titrator, model TTTlc), which continuously monitored the pH of the incubation mixture and controlled the delivery of a solution of 0.3 *M* $NaHCO_3$ and 0.08 *M* glucose. Temperature was maintained at 37°, the mixture was continuously stirred, and the atmosphere in the chamber flushed with a 95% $O_2$–5% $CO_2$ mixture. At the outset and at various times during the incubation procedure, aliquots of the cell suspension were removed for the determinations indicated below.

*Electron microscopy:* The cell suspension was added to 20 volumes of cold 4% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.3, and allowed to fix for 4–6 hr. The cells were then washed with this buffer, and further fixed in 1% osmium tetroxide in 0.1 *M* phosphate buffer pH 7.3, dehydrated, and embedded in epoxy resin. Sections were stained with 1% uranyl acetate in 50% ethyl alcohol and examined in an RCA EMU-2 electron microscope. The total number and arrangement of ribosomes within an arbitrary but standard area inscribed upon micrographs of 50 randomly selected cells was determined. The proportion of cells which appeared devoid of any ribosomes was ascertained. The results of enumeration of ribosomes in duplicate samples differed by less than 5%. The thickness of the sections examined in the electron microscope varied between 400 and 600 Å. A certain fraction of polyribosome structures will be transected by the plane of sectioning and will appear to contain fewer than the actual number of ribosomes. As a consequence of this type of error, the number and size of polyribosomes will be underestimated while the number of single ribosomes will be overestimated. The magnitude of this error was evaluated by an analysis of electron micrographs inscribed with lines simulating adjacent planes of section. This procedure indicated that, on the average, 15% of the ribosomes appear as single particles in these micrographs, but are actually a part of polyribosomes in the cell.

Glutaraldehyde-fixed and washed erythrocytes were also examined in suspension by phase-contrast microscopy and the relative number of mature, bidiscoidal cells estimated.

*Conditions for determination of $C^{14}$-amino acid incorporation by the cells*: The cells were recovered from an aliquot of the incubation mixture by centrifugation and resuspended in a medium containing either $C^{14}$-L-valine or $C^{14}$-L-leucine as described elsewhere.[5] Incubation was for 15 min at 37°. Following this labeling procedure, the cells were recovered by centrifugation and lysed by a short exposure to a hypotonic solution ($1.5 \times 10^{-3}$ *M* $MgCl_2$, $10^{-2}$ *M* Tris-chloride buffer, pH 7.5). This technique preserves leukocytes and tends to disrupt only the red cells.[5] Ribosomes were prepared from the cell lysate by centrifugation at 105,000 × *g* for 120 min. The supernatant was removed and saved (105,000 × *g* supernatant). The pellet of ribosomes was rinsed three times with a solution of $10^{-3}$ *M* Tris-chloride buffer, pH 7.5, $1.5 \times 10^{-3}$ *M* $MgCl_2$ and $5 \times 10^{-2}$ *M* KCl (solution A). The ribosomes were suspended in solution A, and the absorbancy, measured at 260 mμ and 280 mμ, and the radioactivity of the solution were determined.[5] The extinction coefficient of the ribosomes in solution A at 260 mμ was 11.1 optical density units per mg ribosome.

Ribosomes were characterized by their sedimentation properties in sucrose density gradients.[5, 9] The conditions of centrifugation are indicated in the legend for Figure 1. The 105,000 × *g* supernatant was centrifuged for a second time at 105,000 × *g* for 120 min. The supernatant of this second centrifugation was recovered, and aliquots were taken for determination of hemoglobin and protein concentrations, as well as radioactivity incorporated into the protein.[5]

*Other determinations:* At each time when aliquots were removed for electron microscopy and for incubation with the $C^{14}$-amino acid, the following determinations were also made: enumeration of erythrocytes, reticulocytes, nucleated red blood cells and leukocytes,[10] hematocrit,[10] and osmotic fragility of the cells.[11]

*Results and Discussion.—Distribution of ribosomes in reticulocytes:* An estimate of the proportion of ribosomes arranged in polyribosome structures in the intact cell was obtained from examination of electron micrographs of sectioned reticulocytes. Such an analysis revealed that 80–90 per cent of the ribosomes were polyribosomes within the population of reticulocytes at the outset of *in vitro* maturation (Table 1). This value was somewhat higher than the proportion of ribosomes sedimenting with a sedimentation coefficient greater than 78S as estimated by the sucrose density gradient method. Among the possible explanations for finding a lower proportion of ribosomes sedimenting with a sedimentation coefficient greater than 78S, as compared with the ribosomes appearing as polyribosomes in sectioned cells, are (1) that a fraction of the polyribosomes are disrupted in the preparation of ribosomes for analysis by sucrose density gradient technique, and (2) that the procedure of lysis of the reticulocytes does not recover the polyribosomes as completely as the 78S ribosomes. Previous studies have demonstrated that polyribosomes may be broken down to 78S particles by such procedures as homogenization during resuspension of the ribosome pellet, repeated centrifugation, or exposure to high salt concentrations.[5, 6, 12] The limited manipulations involved in the preparation of the electron micrographs suggests that the results obtained by this type of analysis more accurately reflect the proportion of the ribosomes which are polyribosomes in the intact reticulocyte.

TABLE 1

PROPORTION OF RETICULOCYTE RIBOSOMES THAT ARE POLYRIBOSOMES

| | Method | |
|---|---|---|
| Rabbit* No. | Electron microscopy % polyribosomes† | Sucrose density gradient % > 78S‡ |
| I | 91 | 75 |
| II | 83 | 76 |
| III | 81 | 65 |
| IV | 90 | 75 |
| V | 84 | — |
| VI | — | 66 |

* All rabbits had 55–80% reticulocytes in the red cell population.

† The proportion of the total number of ribosomes which appeared in clusters of 2 or more. These values are corrected for the estimated number of polyribosomes transected by the plane of sectioning as described in *Methods*.

‡ The proportion of the ribosomes sedimenting with sedimentation coefficients greater than 78S was estimated by integration of the area under the peaks in the pattern of distribution of ribosomes after sedimentation in the sucrose density gradient.

*Effect of in vitro maturation on ribosome clusters and protein synthesis:* The occurrence of reticulocyte maturation in the *in vitro* system employed was evidenced by a decrease in the percentage of cells that stained as reticulocytes, an increase in the relative number of mature, bidiscoidal cells as evaluated in phase microscopy, and an increase in the osmotic fragility of the cells.

In each of six experiments, *in vitro* maturation was associated with a decrease

in the rate of amino acid incorporation into soluble protein and in the amount of labeled amino acids associated with ribosomes (Table 2). The kinetics of amino acid incorporation by cells at the outset and following *in vitro* maturation were examined. The incorporation of amino acids associated with the ribosomes reached a plateau level within two min which persisted for as long as one hr. During this time, amino acid incorporation into soluble protein continued to increase at a linear rate. In each study (Table 2) the decrease in the *rate* of amino acid incorporated into soluble protein was comparable to the fall in the level of newly incorporated amino acid associated with ribosomes. This finding is consistent with the concept that the decrease in protein synthesis is due to a decrease in the cell content of ribosomes active in protein formation.

TABLE 2

EFFECT OF RETICULOCYTE MATURATION *in vitro* ON PROTEIN SYNTHESIS AND RIBOSOME CONTENT

| Experiment | *In vitro* maturation time (hr) | Amino Acid Incorporation (mμmoles/$10^{10}$ cells)* | | Ribosome Content† | | | |
|---|---|---|---|---|---|---|---|
| | | | | Electron microscopy (no./unit area of 50 cells) | | Sucrose density gradient (mgm) | |
| | | Ribosomes | Soluble protein | Total | Clustered | Total | >78S |
| I | 0 | 8.10 | 316 | 2,242 | 2,040 | 4.4 | 3.3 |
| | 24 | 0.76 | 28 | 1,485 | 1,100 | 3.0 | 1.4 |
| II | 0 | 5.01 | 212 | 685 | 570 | 2.6 | 2.0 |
| | 24 | 0.15 | 5 | 133 | 57 | 0.6 | 0.2 |
| III | 0 | 5.70 | 280 | 1,717 | 1,390 | 4.8 | 3.6 |
| | 22 | 1.62 | 76 | 702 | 619 | 1.4 | 1.0 |
| IV | 0 | 8.5 | 354 | 2,744 | 2,290 | 5.9 | ND‡ |
| | 21 | 1.9 | 70 | 957 | 440 | 2.0 | ND |
| V | 0 | 6.4 | 391 | ND | ND | 4.2 | ND |
| | 24 | 0.6 | 35 | ND | ND | 2.1 | ND |
| VI | 0 | 9.6 | 428 | ND | ND | 4.2 | 2.7 |
| | 21 | 1.6 | 67 | ND | ND | 1.6 | 0.9 |

* During the 15 min incubation.
† Determined by enumeration of the ribosomes in electron micrographs of sectioned cells and as estimated from analysis of the patterns of sedimentation in sucrose density gradients.
‡ ND indicates determination not performed.

More direct evidence that the fall in protein synthesis as reticulocytes mature is related to a decrease in ribosome clusters is presented in Figures 1 and 2. Ribosomes isolated from reticulocytes before and after *in vitro* maturation were analyzed by centrifugation in a sucrose density gradient. With maturation the proportion of ribosomes which sediment with a sedimentation coefficient greater than 78S decreases, while the proportion of 78S ribosomes increases (Fig. 1). The decrease in the polyribosomes with reticulocyte maturation was further established by examination of electron micrographs of sectioned cells. At the outset of maturation, ribosomes are observed predominantly in clusters of 2 or more (Fig. 2*A*). With *in vitro* maturation, there is a fall in the total number of ribosomes and an even more striking decrease in the proportion of the ribosomes which are polyribosomes (Table 2, Fig. 2*B*). The polyribosomes which remain show an alteration in the pattern of distribution characterized by a shift toward smaller clusters, as well as an increase in the proportion of single ribosomes (Fig. 3). The decrease in the total ribosome content of cells following maturation is consistent with the observations of Bertles and Beck[13] that rabbit reticulocyte maturation *in vitro* is accompanied by a conversion of cellular ribonucleic acid to low molecular weight products, chiefly purines and pyrimidines.

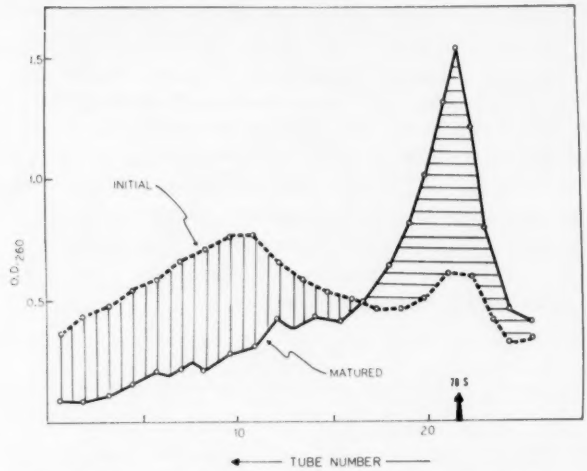


FIG. 1.—Effect of *in vitro* maturation of reticulocytes on the distribution of ribosomes of various sizes. Sucrose density gradient analysis of ribosomes isolated from reticulocytes before (broken line) and after (solid line) a 22-hr period of *in vitro* maturation. The ribosomes were suspended in a solution of $10^{-3}$ *M* Tris-HCl buffer, pH 7.5, $1.5 \times 10^{-3}$ *M* $MgCl_2$, and $5 \times 10^{-2}$ *M* KCl, and sedimented by centrifugation in a 5–20% sucrose density gradient prepared from the same solution. Centrifugation was for 2 hr at 25,000 rpm in a Spinco SW-25 rotor. The gradient was analyzed as previously described.[5]

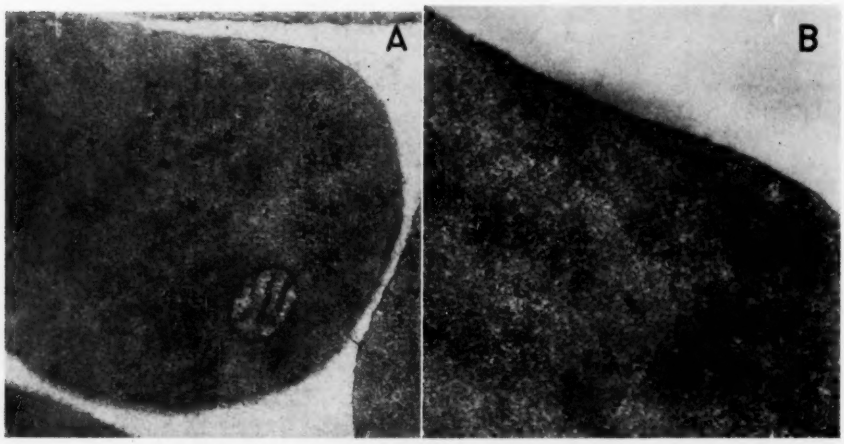


FIG. 2.—(*A*) A portion of two reticulocytes at the outset of *in vitro* maturation. Numerous ribosomal clusters are observed in a finely granular, dense material presumed to be hemoglobin. A mitochondrion is present in the cell. (*B*) Part of a recticulocyte following 24 hr of *in vitro* maturation. The cytoplasm contains many ribosomes, but these are virtually all single nonclustered particles. (Magnification 38,000 ×).

The ribosomes involved in protein synthesis were examined by determining the pattern of incorporation of $C^{14}$-leucine onto ribosome clusters of various sizes. At both the initial time and following *in vitro* maturation, the specific activity of the ribosomes in the region corresponding to sedimentation coefficients greater than

100S was markedly higher than that of 78S ribosomes (Fig. 4). These data demonstrate that, despite marked loss in the capacity to form protein, protein synthesis which continues to occur proceeds predominantly on the polyribosomes. In 3 of 4 experiments, a decrease in the specific activity of polyribosomes occurred during *in vitro* maturation (Table 1 and Fig. 4). Thus, as reticulocytes mature, there is a fall in the capacity of polyribosomes to incorporate amino acids into polypeptides, as well as a decrease in the amount of polyribosomes. This finding suggests that polyribosomes may become partially or completely inactive while remaining polyribosomes. One of the possible explanations for these findings is that the loss of polyribosomes occurs only after messenger RNA or another component of the protein synthetic mechanism is depleted during reticulocyte maturation.

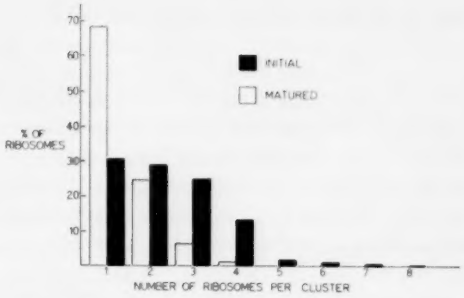


FIG. 3.—Effect of maturation on the distribution of ribosomes in various size clusters as determined by analysis of electron micrographs of sectioned cells. The values before (open bars) and after (closed bars) *in vitro* maturation are expressed as per cent of the total number of ribosomes per standard area of 50 cells. These values are not corrected for the error introduced by sectioning because the redistribution of ribosomes among each of the classes of polyribosomes cannot be made with accuracy.

It has been proposed that the polyribosomes are composed of a number of 78S ribosomes attached to a strand of messenger RNA.[6, 7, 14–16] Although evidence has been obtained consistent with this hypothesis,[5–7, 14–21] there has been no

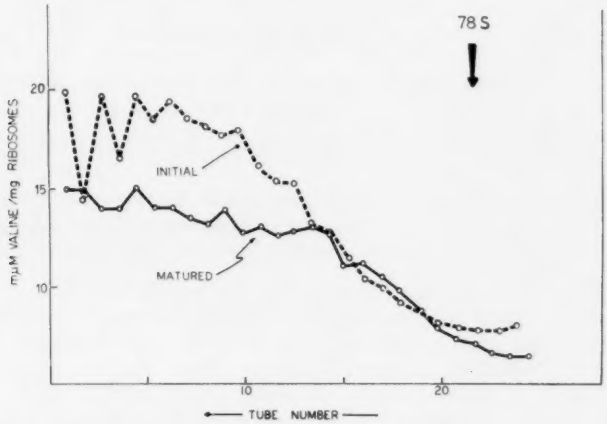


FIG. 4.—Effect of *in vitro* maturation of reticulocytes on the pattern of amino acid incorporation on ribosomes of various sizes. The reticulocytes were incubated with $C^{14}$-leucine under the conditions described in *Methods*. The preparation of ribosomes and the conditions for sucrose density analysis were identical to those indicated in the legend for Fig. 1. The pattern of incorporation of $C^{14}$-leucine on the ribosomes is expressed as mμmoles leucine incorporated per mg of ribosomes before (broken line) and after (solid line) *in vitro* maturation.

definitive demonstration that the substance essential for the maintenance of polyribosome structure is totally accounted for by a messenger RNA, i.e., a fraction of RNA which has the biological property of a template for protein synthesis. In reticulocytes, there is no detectable synthesis of any RNA component of the polyribosome.[5] The stability of these polyribosomes is therefore dependent on a factor or factors other than continued RNA synthesis. There is evidence that a portion of ribosomal RNA,[22] as well as transfer RNA,[21] may be important to the stability of the polyribosomes. The present observations suggest that the maintenance of the polyribosome structure is a fundamental aspect of the regulation of erythroid cell maturation.

*Summary.*—In a population of relatively immature rabbit reticulocytes, 80–90 per cent of the ribosomes appear as polyribosomes in electron micrographs of sectioned cells. Evidence has been obtained indicating that the maturation of reticulocytes to erythrocytes is associated with a decrease in the functional capacity, as well as in the number of polyribosomes which are the site of protein formation. It is likely that these alterations in polyribosomes account for the decrease in protein synthesis which occurs as reticulocytes mature.

The authors are indebted to the Department of Microbiology and, in particular, Dr. Councilman Morgan for making available the facilities of the electron microscope laboratory.

* This investigation was supported in part by grants from the U.S. Public Health Service and the National Science Foundation.

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# *RAPID TRANSFORMATION OF HUMAN FIBROBLAST CULTURES BY SIMIAN VIRUS 40**

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*Communicated by Dr. Warren H. Lewis, June 26, 1963*

Cultures of normal human fibroblasts are characterized by a finite lifetime *in vitro*.[1] Actively proliferating cells can usually be maintained in culture for only 35–40 transfers. Following this period the growth rate of the cultures gradually declines, and proliferation ceases completely after 5–10 further passages.[1, 2]

In previously reported studies[3–6] on transformation of human cell cultures by simian virus 40 (SV40), either primary explants or actively proliferating established cell strains were exposed to SV40. In this study the susceptibility to SV40 of cultures approaching the end of their expected *in vitro* lifetime was assessed. It was found that these aging cultures transformed considerably earlier after infection than the primary explants and "young" actively proliferating cell strains examined before.

*Materials and Methods.*—The WI-26 and WI-38 human fibroblast strains were originally obtained by cultivation of embryonic lung tissue.[1] Cells of two WI-26 substrains used in the present study (Nos. XII and XXVI, Table 1) could not be transferred beyond the 41st and 56th subcultures, respectively. Each of three parallel cultures of substrain XXVI was exposed to SV40 at the 55th subculture. Cells of the parent WI-38 strain and of its No. IV substrain did not survive subculturing beyond the 50th and 48th passages, respectively. As shown in Table 1, these cultures were exposed to SV40 at the level of the 50th and 47th subcultures.

The W-5 CM strain originated from an explant of a fragment of buccal mucosa obtained from a 72-year-old man who had been treated with X rays and cytoxan after surgical removal of a bronchial carcinoma.[3] Following formation of a monolayer, the cell strain was propagated in actively proliferating cultures up to the 23rd transfer, when the cultures were frozen and stored, but could not be recovered in a viable state after thawing.

Two substrains (D 90 and D 285) were established from the 12th passage of the parental W-5 CM strain after the culture flasks were left in an incubator for 90 and 285 days, respectively, without a change of nutrient medium. Although this prolonged incubation period led to a cessation of mitosis and some cell loss, the remaining cells of the two cultures started to proliferate again when fresh medium was supplied. However, cells of substrain D 90 could be subcultured only at weekly intervals as compared to biweekly transfers for the parent strain. The cell growth of D 90 slowed down even more at the level of the 19th passage, and the cultures could not survive beyond the 25th–30th passage (Table 1). Each of the three parallel cultures of this substrain was exposed to SV40 at the 21st, 21st, and 22nd passages, respectively (Table 1).

Three parallel cultures of substrain W-5 D 285 (Table 1) were infected with SV40 at the 13th passage, i.e., the first passage following restoration of cultures after prolonged incubation. Although parallel noninfected cultures of this strain are still maintained, growth is so slow that they can be subcultured only at intervals of 2–3 weeks.

TABLE 1

TRANSFORMATION OF HUMAN FIBROBLASTS EXPOSED TO SV40 AT DIFFERENT PASSAGE LEVELS *in vitro*

| Characteristics of the Cultures | | | | |
|---|---|---|---|---|
| Strain | Substrain | Maximum number of passages obtained with noninfected parallel cultures | Passage at time of exposure | Time interval between exposure and morphologic transformation (weeks) |
| WI-26 | XII | 41 | 27* | 10 |
| | XXVI | 56 | 55 | 5 |
| | | 56 | 55 | 5 |
| | | 56 | 55 | 3 |
| WI-38 | Parental | 50 | 9* | 8 |
| | | | 25* | 8 |
| | | | 50 | $4^1/_2$ |
| | IV | 48 | 47 | $4^1/_2$ |
| W-5 CM | Parental | >23† | Primary explant‡ | 9 |
| | D 90 | 25 | 21 | 4 |
| | | 27 | 21 | 3 |
| | | 30 | 22‡ | $2^1/_2$ |
| | | | 13 | 3 |
| | D 285 | | 13 | $3^1/_2$ |
| | | | 13‡ | 4 |

* Observation previously reported.[6]
† See text.
‡ Green monkey kidney pool of SV40 used for infection; in all other cases human tissue pool of virus was used.

Two pools of SV40 virus were used for infection of tissue cultures—one prepared in green monkey kidney, and the other obtained from chronically infected transformed cultures of human cells.[6] General handling of normal and infected cultures and techniques used for morphological examinations did not differ from those reported before.[3, 6] Chromosome analysis was performed on cell samples obtained from cultures at different passage levels before and after exposure to SV40. The chromosome preparation technique used in this study has been described in detail elsewhere.[2, 7]

*Results.*—Results shown in the 3rd and 4th columns of Table 1 indicate that many of the cell strains were indeed at the end of their *in vitro* lifetime at the time of their exposure to SV40. Their rate of growth was very slow, and there was virtual absence of mitotic figures. However, within two weeks after infection, the appearance of the cultures began to change. Mitotic figures and foci with increased cell density were clearly visible, and a slight cytopathic effect was noted. None of these changes was observed in noninfected control cultures.

Morphological transformation occurred within two weeks after the first increase in mitotic rate was observed. Definite transformation was considered to have taken place when the following three criteria were met: (1) "loss of mitotic inhibition," i.e., presence of numerous mitotic figures even in areas of confluent cells; (2) disorganized multilayer growth; and (3) occurrence of epithelial-like cells. This corresponds to "early transformation," as defined previously.[6] From the "early transformation" stage the cultures progressed to a "complete transformation."[6]

As shown in Table 1, the time lapse between exposure to SV40 and transformation was much shorter for the three cultures of WI-26 substrain XXVI which transformed 3–5 weeks after infection, as compared to the XII substrain, which, when exposed at the level of the 27th passage during a period of active proliferation, transformed ten weeks after exposure to the virus.[6] Cultures of the parent WI-38

strain, which were also exposed at the time of their active proliferation (9th and 25th passages), transformed eight weeks after infection, whereas cultures of the parent and No. IV substrain, infected at the end of their *in vitro* lifetime, transformed after $4^1/_2$ weeks. The same holds true for D 90 and D 285 substrains of the W-5 CM culture which transformed $2^1/_2$–4 weeks after infection, in contrast to the parental culture which transformed nine weeks after exposure as a primary explant.[3, 6]

The "rapid" transformation process was also characterized by development of a much sharper contrast between the transformed and the nontransformed areas than noted in the "slow" transformation process. This was presumably observed because cells in the nontransformed areas were in a resting state in contrast to the areas of rapidly dividing transformed cells.

In the course of only one passage, subcultivation of the transforming cultures in the present series resulted in the establishment of virtually pure cultures of transformed cells, since presumably nontransformed cells were unable to divide.

Apart from these differences, the transformation process was morphologically identical, regardless of whether the cultures were infected by SV40 in a stage of active growth or in a stationary or regressive phase.

Following exposure to either of the two virus pools (see above), all transformed cultures became infectious. Results of preliminary titrations indicate that the yield of virus was greater from cultures which had undergone the "rapid" transformation process than from those having undergone "slow" transformation.

*Chromosome studies:* Detailed karyotype analysis of the WI-26 and WI-38 strains is the subject of a paper by Saksela and Moorhead.[2] In brief, chromosome counts of 200 WI-26 and 400 WI-38 cells of different substrains revealed no departure from classic diploidy *before* the level of the 37th–41st subcultures was reached. Thus, at the time of the earliest exposure to SV40 (27th passage for WI-26, XII and 9th for WI-38, P; Table 1), the cultures were chromosomally normal. During the declining phase of culture life, *after* the 40th passage, the WI-26 and WI-38 cells did show spontaneous aneuploidy characterized by various chromosomal aberrations in 2–30 per cent of the dividing cells, but monosomy of the 21–22 group was not a prominent feature of these abnormalities. None of the WI-26 and WI-38 substrains characterized by partial aneuploidy[2] and none of several other substrains observed during prolonged growth *in vitro* has undergone spontaneous transformation.

A karyotype of the cells of the buccal mucosa of patient W-5 was not determined. However, in the noninfected cultures at the level of the 7th subculture, 13 out of 58 cells examined were monosomic for a 21–22 chromosome.[3, 7] The proportion of monosomic cells increased during further cultivation, as indicated by the fact that 41 out of 47 cells at the level of the 17th–19th passages and all of the 16 cells examined at the level of the 20th passage were characterized by the absence of one chromosome from the 21–22 group.[7]

The cells of the W-5 substrains (Table 1) were also characterized by monosomy for the 21–22 chromosome, the proportions being about 80 per cent for D 90 and about 50 per cent for D 285, respectively.

Four weeks elapsed between the observed morphologic transformation after SV40 infection and the first appearance of high frequency of dicentrics, minutes, and other obvious chromosome changes in substrain XXVI of WI-26. The time period between these two events was identical to that observed in the "slow" transformation process of cultures infected in an actively proliferating phase.

*Discussion.*—The exact mechanism of the transformation process of tissue cultures by tumor-inducing viruses is essentially unknown. Therefore, it would be difficult to find an easy explanation for the unusually rapid transformation of human cell cultures infected with SV40 at the end of their *in vitro* lifetime, although it would be tempting to link together these two phenomena, the age of the culture and the "transformation time" (the time from exposure of the cultures to their morphologic alteration). Before this will be considered, however, thought should be given to the possibility that the low metabolic activity of cells unrelated to their age *in vitro* predisposes the culture to a more rapid transformation with SV40.

This hypothesis which stresses the physiologic state of the culture rather than the "genetic" makeup of its cell population can be put to test in SV40 transformation experiments in which proliferation of cells will be artificially inhibited during any phase of cultivation. However, the results of Shein and Enders[4] suggest a lack of relationship between the low metabolic activity of cells and the "transformation time."

If the low metabolic activity *per se* of cells in culture does not predispose them to a more rapid transformation, then the relationship between the process of aging *in vitro* and susceptibility to transformation with SV40 should be given more prominent consideration. Within this context it may be postulated that the target for SV40 transformation is a "defective" cell which is predominant in the cell population of a culture at the end of its lifetime, but represents only a small fraction of cells in an actively proliferating culture. This "defective" cell has a low capacity for proliferation and its "defectiveness" would be expressed as aneuploidy when such cells do undergo mitosis. Aneuploidy was prevalent in the aged cultures of WI-26 and WI-38 strains.[2] Infection with SV40 would restore the proliferative capacity of the "defective" cells and set in motion the process of transformation.[6] This fact is compatible with the known effect of infection with SV40, i.e., stimulation of mitosis either by direct action on the cell or by suppression of normally occurring mitotic inhibition. This characteristic activity, which has been observed in cultures of human and hamster fibroblasts prior to their morphologic transformation, is not limited to these normal cell systems since proliferation of HEp-2[8] and HeLa[8, 9] cells, and of polyoma virus-transformed hamster cells was also markedly enhanced after infection with SV40.[9]

If in actively proliferating cultures the number of "defective" cells is small, then the time between stimulation of the mitotic apparatus by infection with SV40 and proliferation of the infected cells to a point of formation of morphologically distinguishable colonies may be long. This may be particularly true if the growth potential of the "nontransforming" cells of the same culture is high, as the case may be for a "young" culture of human fibroblasts. Conversely, in aging cultures "defective" cells present in large number may need to undergo only a few cell

divisions after stimulation by SV40 infection before the morphological changes will be detected in the culture.

The role of the missing 21–22 chromosome in relation to the transformation process is difficult to evaluate. Deletion of this particular chromosome, one of the two small acrocentrics, has already been observed as an early change in the transformation process after infection with SV40 virus.[4, 7] It cannot yet be said whether this deletion is a specific phenomenon or occurs only as the result of a selective advantage exhibited by cells monosomic for a small acrocentric, as compared to cells in which the disturbance of karyotype is more drastic. Obviously, this monosomy is not a sufficient condition for transformation since many uninfected W-5 CM cultures have not transformed, although observed for a long period of time.

It is possible that "defective" cells are not present in primary explants but appear *in vitro* as the result of conditions leading to mutations among the proliferating cells (background irradiation, heterologous sera, etc.). The "defective" cell may also arise as the result of the presence of a "virus-like agent" which, on the one hand, may be indirectly responsible for the finite lifetime of cultures but, on the other hand, may facilitate transformation by SV40.

Interaction between SV40 and an "unknown" virus has few parallels in other animal virus systems except perhaps in the case of nodule-inducing (NIV) and mammary tumor viruses (MTV) which, as postulated by De Ome,[10] have to interact in order to cause mammary adeno-carcinoma in mice.

The difference between this system and the SV40-induced transformation lies in the fact that particles which may represent NIV were observed under electron microscopy,[10] whereas there is no known test for the presence of any virus which predisposes the mammalian cells to transformation by SV40.

The possibility cannot be excluded that the presence of such an agent can be demonstrated if SV40-infected actively proliferating cultures would transform more rapidly after exposure to extracts from "defective" cells or after previous cell-to-cell contact with their cultures.

Finally, it is possible, although not very probable, that infection of either aging or stationary cultures leads to selection of particles of SV40 endowed with a character for "rapid" transformation. This hypothesis is being tested by the exposure of actively proliferating cultures to preparations of virus obtained from cultures infected in a stationary phase. Results of experiments performed to date do not support this theory.

The authors are deeply indebted to Dr. Paul S. Moorhead and Dr. Eero Saksela for their extensive contribution in chromosome studies and valuable discussions associated with this manuscript. They also wish to thank Mr. Stanley Cholodofsky, Mr. Joseph Higgs, and Mrs. Jean Wilson for their excellent technical assistance.

* This work was supported (in part) by USPHS research grant CA-04534, contract no. PH-43-62-157, and career development award 5K3-CA-18,372 from the National Cancer Institute.

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## *PROPERTIES OF A TEMPERATURE-SENSITIVE REGULATORY SYSTEM**

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*Communicated by Herman M. Kalckar, June 14, 1963*

The synthesis of a number of inducible and repressible enzymes in bacteria has been shown to be controlled by the interaction of specific metabolites—inducers and "corepressors"—with specific, cytoplasmically diffused gene products which have been termed "aporepressors."[1] Little is known, however, about the nature of the postulated aporepressors, the molecular bases of their specificities, or the level(s) at which they intervene in the transcription of the information of structural genes.

In principle, some light might be shed on these questions through physiological experiments. Accordingly, numerous workers have examined the effect on enzyme repression of various regimes of unbalanced growth.[2] The ambiguities of these studies probably stem from two features of enzyme repression: (1) the synthesis of many inducible or repressible enzymes is subject to a complex and little understood repressive effect of carbon source dissimilation;[3] physiological manipulations which affect the pattern of catabolism consequently produce profound secondary effects on the rate of synthesis of regulated enzymes (e.g., the effect of thymine starvation on beta-galactosidase induction).[4] (2) If aporepressor molecules have a very high affinity for their sites of action[5] or a very high rate of synthesis,[6] then the sensitivity of the regulatory system may be too slight, or its response too transient, to be detectable. In particular, if aporepressors are rapidly synthesized and rapidly turned over, inhibitor studies would be complicated by the speed with which the steady state level of aporepressor will return to normal after the removal of inhibition.[6]

As a result of these considerations, we have sought a regulatory system in which the difficulties described above might be avoided. Such a system should: (1) be insensitive to catabolite repression, and (2) have a damaged regulatory system, hopefully one in which the rate of synthesis of aporepressor could be experimentally controlled. The synthesis of alkaline phosphatase in *E. coli* seems to satisfy condition (1), in that it is insensitive to catabolite repression[4, 7] while subject to a highly specific and genetically defined end product repression.[8] Consequently, we isolated a mutant which showed temperature-sensitive repression of alkaline phosphatase synthesis.[9] The present communication deals with the kinetics of phosphatase synthesis in this strain. Our observations indicate that the temperature sensitivity applies to the rate of synthesis of aporepressor. Variation in this rate

with temperature enables us to detect the fact that the aporepressor is metabolically unstable, with a time constant for inactivation during growth of about 0.2 generations, independent of temperature.

*Methods and Materials.*—Strain B3 is a thymineless mutant of *E. coli* B. PR1 is a 5-bromouracil induced mutant of B3 which shows temperature-dependent constitutivity of alkaline phosphatase synthesis[9] (Fig. 1). The medium employed in all experiments was Davis' minimal,[10] except that potassium phosphate was present in a concentration of $2 \times 10^{-3}$ *M* (excess phosphate) or less, as specified, and Tris (0.05 *M*, pH 7.4) was employed as buffer. Thymine was always present at 10 $\mu$g per ml. Unless otherwise indicated, the carbon source was 0.2% glucose.

For enzyme assays, culture samples were centrifuged, resuspended in 0.1 *M* Tris, pH 7.4, and toluenized for 60–90 min at 37°. Alkaline phosphatase and beta-galactosidase were assayed according to Echols *et al.*,[8] except that the substrate concentration in the phosphatase assay was reduced to 1 mg/ml. Dihydroorotic acid dehydrogenase was assayed according to Yates and Pardee.[11] For each enzymatic activity, one unit corresponds to a change of optical density, at the appropriate wavelength, of 1.00 per min at 25°.

Cell density was measured as optical density at 720 m$\mu$. Specific activities (and differential rates) are reported as enzyme activity divided by optical density at 720 m$\mu$. An OD of 1.00 at 720 m$\mu$ corresponds to 220 ± 20 $\mu$g protein per ml under these conditions.

DNA, RNA, and protein were measured by conventional methods described elsewhere.[12]

*Results and Discussion.*—Figure 1 illustrates the temperature dependence of repression of PR1. The specific activity of alkaline phosphatase in limiting phosphate, as well as the specific activities of two control enzymes, is seen to be essentially temperature-independent. On the other hand, the specific activity of phosphatase in excess phosphate varies over a 300-fold range. At seven temperatures between 25° and 39°, we have verified that the specific activity observed after glucose exhaustion corresponds to a differential rate of synthesis during exponential growth. It should be noted that a limiting degree of repressibility is reached at temperatures below about 21°, the specific activity in excess phosphate dropping no lower than 0.002–0.003 units. This level is still somewhat above the specific activity of the fully repressed parental strain, which is 0.0005 ± 0.0002 independent of temperature.

The constitutivity of alkaline phosphatase synthesis at high temperatures is attributable either to an intracellular deficiency of corepressor, inorganic phosphate, or to a deficiency of aporepressor function. In PR1's repressible parent strain B3, derepression commences at growth-limiting concentrations of phosphate, as it does in strain K12.[7, 13] Therefore, an intracellular deficiency of phosphate sufficient to derepress alkaline phosphatase synthesis should also produce a decrease in growth rate. On the contrary, we find that in excess phosphate the growth rates of PR1 and of B3 are identical at 39° and 37°, as well as at 25°. A more critical test of phosphate uptake would be the dependence of growth rate on extracellular phosphate concentration. Consequently, we have compared in detail the growth of PR1 and B3 at phosphate concentrations low enough to be exhausted during growth. Such an experiment, which is shown in Figure 2, measures growth re-

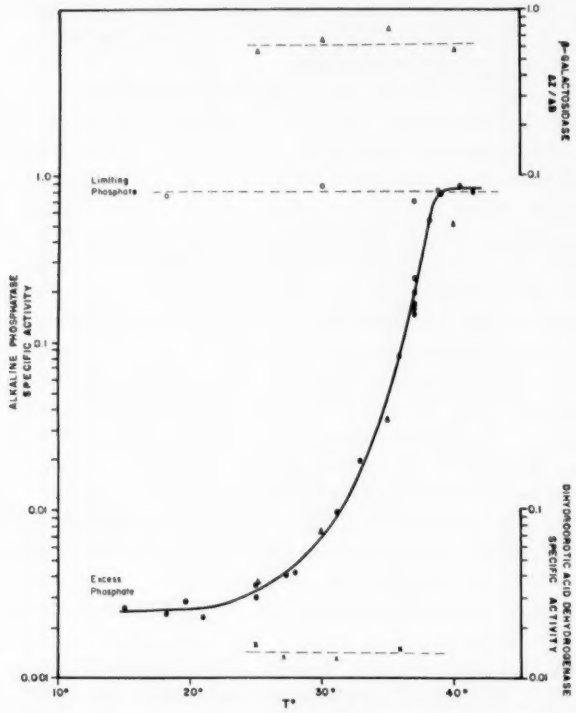


FIG. 1.—Temperature dependence of enzyme synthesis in PR1. Open circles: specific activity of alkaline phosphatase in overnight cultures grown in limiting phosphate ($5 \times 10^{-3}$ *M*). Filled circles: specific activity of alkaline phosphatase in cultures grown overnight in excess phosphate with limiting glucose (0.02%). Filled triangles: differential rate of alkaline phosphatase synthesis in excess phosphate with 0.4% glycerol. Open triangles: differential rate of induced synthesis of beta-galactosidase in excess phosphate, 0.4% glycerol, $10^{-3}$ *M* TMG. X's: specific activity of dihydroorotic acid dehydrogenase in cultures grown overnight in excess phosphate, limiting glucose.

sponse in a continuously decreasing concentration of phosphate.[7] Macromolecular synthesis responds in a characteristic way, as Horiuchi *et al.*[14] have described: a strikingly sharp break in the rate of increase of turbidity, an equally sharp break in RNA synthesis followed by RNA breakdown, and a more gradual decrease in the rate of protein and DNA synthesis. Figure 2 shows that the time course of these events is virtually identical in cultures of PR1 and B3 at 39°. It is highly implausible that at 39° PR1 maintains an internal phosphate concentration low enough to derepress alkaline phosphatase synthesis almost completely, yet high enough to maintain RNA, DNA, and protein synthesis at their maximum rates, and furthermore that it does so, independently of the extracellular phosphate concentration. We conclude that the temperature-sensitive variable in PR1 is the level of active aporepressor rather than the availability of corepressor.

The data of Figure 1 present the specific activity of alkaline phosphatase and its differential rate of synthesis under equilibrium conditions, i.e., after many generations of growth at each temperature. When a culture growing exponentially

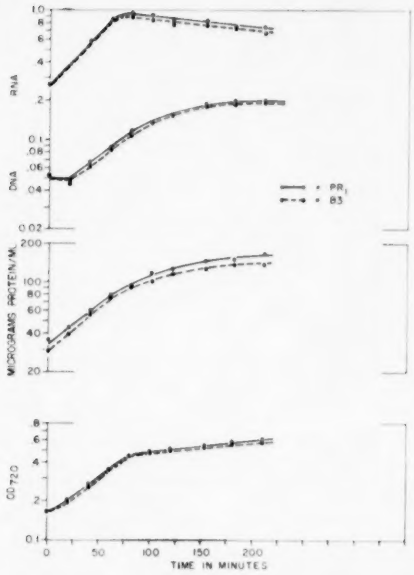


FIG. 2.—Growth of PR1 and B3 in $8 \times 10^{-5}$ *M* phosphate at 39°. Cultures of B3 and PR1 growing in excess phosphate at 39° (differential rates of phosphatase synthesis were 0.0004 and 0.71, respectively) were harvested, washed in phosphate free medium, and cultivated at 39° in medium containing $8 \times 10^{-5}$ *M* phosphate. DNA and RNA are shown on the same scale in arbitrary units. One arbitrary unit corresponds to an optical density of 1.00 at 260 mμ after hydrolysis in 0.5 *N* perchloric acid.

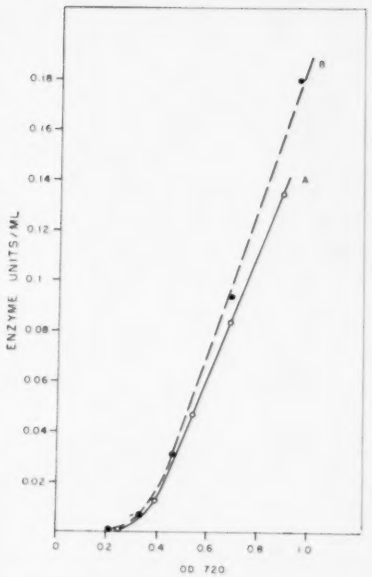


FIG. 3.—Alkaline phosphatase synthesis after temperature shift. A culture growing exponentially in excess phosphate at 25° was harvested, washed, and resuspended in the excess phosphate medium. Portion A was cultivated at 37° immediately. Portion B was incubated at 37° without glucose for 40 min (about the length of the lag period of enzyme synthesis in culture A) and then growth was initiated by the addition of glucose.

under conditions of repression, at 25°, is shifted to a higher temperature, the synthesis of enzyme responds as shown in Figure 3. The differential rate rises through a distinct lag period to a limiting value characteristic of the temperature. The lag period is too short to be accounted for by dilution of a stable, preformed aporepressor. Therefore, the progressive increase in differential rate during this period must reflect the inactivation of the material responsible for repression before the temperature was raised.

Comparison of curves *A* and *B* in Figure 3 reveals that this inactivation process is a matter of metabolic rather than of chemical instability. Preincubation at the higher temperature without growth (curve *B*) does not decrease the lag period or significantly affect the limiting differential rate subsequently attained. Thus, the inactivation is dependent on growth.

This point is amplified by the experiment illustrated in Figure 4. This experiment shows that, in a culture at temperature equilibrium, the differential rate of synthesis is not significantly affected either by heating in the absence of growth (curve *D*) or by decreasing the rate of growth at constant temperature (curves *B* and *C*).[15]

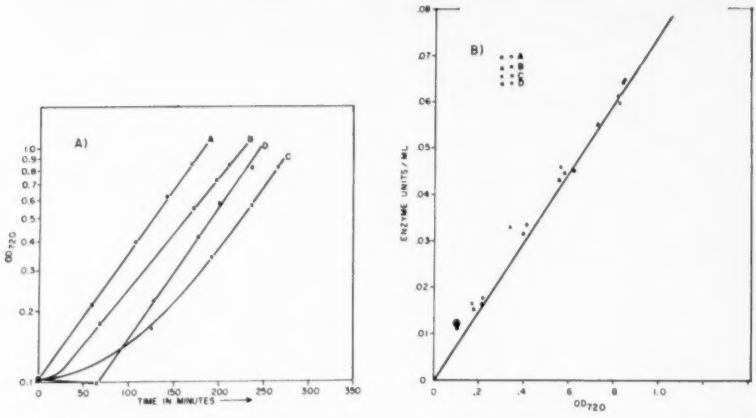


FIG. 4.—Effect of preheating and reduced growth rate. (In this experiment PR1A2, a leucineless derivative of PR1, was used, and leucine at 20 $\mu$g per ml was added to each culture.) A culture growing exponentially in excess phosphate at 35° was harvested, washed, resuspended in excess phosphate medium, and divided into four portions *A*, *B*, *C*, and *D*. *A* was cultivated at 35° on 0.2% glucose as a control. *B* was cultivated on 1% sodium lactate, producing a short lag and a modest (18%) decrease in growth rate. *C* was cultivated on 0.4% lactose, producing a long induction lag during which the growth rate was much lower than that of culture *A*. *D* was incubated without a carbon source (zero growth rate) at 37° for 60 min before return to 35° and addition of glucose to 0.2%. Fig. 4*A* presents the growth of these cultures as a function of time, while Fig. 4*B* shows their enzyme activity as a function of growth.

Two explanations for the growth-dependent inactivation of aporepressor are possible. On the one hand, it may be a process specifically originating from the mutational lesion in PR1. In this case, since repression in PR1 is temperature-dependent, the inactivation reaction should be temperature-dependent. On the other hand, the inactivation may represent a normal metabolic turnover of aporepressor. In this case the mutational lesion, and hence the temperature sensitivity, must apply to the rate of synthesis of aporepressor.

The simplest way to distinguish between these explanations is to determine the temperature dependence of the inactivation reaction. This was done in the experiment illustrated in Figure 5. Portions of a culture growing exponentially at 25° were transferred to 37° and 39° and sampled at frequent intervals throughout the lag period. Figure 6 presents the data of Figure 5 in a more informative way. Here we plot the average differential rate of synthesis during each successive growth interval versus the average OD during that growth interval. This plot shows that the initial rate of increase of differential rate of enzyme synthesis is not significantly different at the two temperatures. Therefore, the inactivation reaction is not temperature-sensitive.[19] Since the equilibrium differential rate is about threefold higher at 39° than at 37°, the temperature-dependent variable must be the rate of aporepressor synthesis.

It is worth noting that this argument is independent of the nature of the inactivation reaction, i.e., whether it were reversible or irreversible. If the temperature sensitivity applied to the inactivation reaction, we would have to assume that the extent of the reaction was temperature-dependent but that its rate was not. This hypothesis requires that the population of aporepressor molecules be heterogeneous,

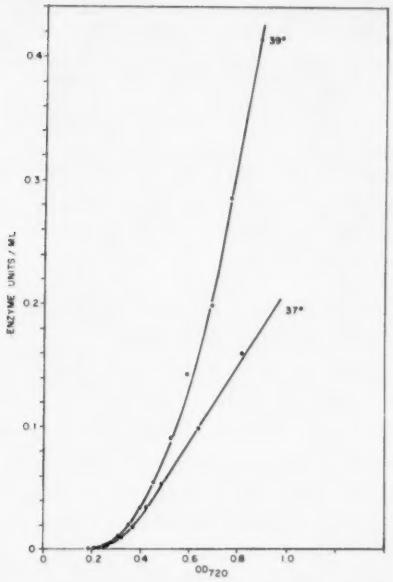


FIG. 5.—Alkaline phosphatase synthesis after temperature shift, two temperatures. A 25° culture was harvested as described above, and cultivated at 37° and 39°.

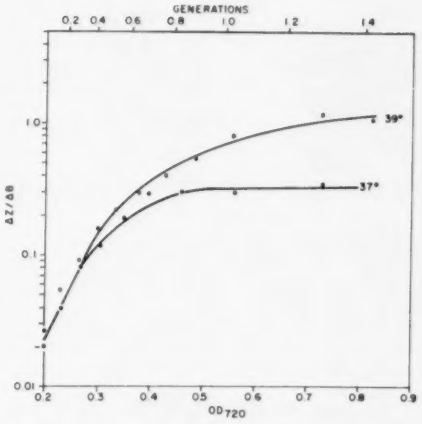


FIG. 6.—Rate of increase of differential rate of phosphatase synthesis. The data of Fig. 5 are replotted as described in the text. The upper abscissa gives generations of growth of each culture, where one generation is defined as the average doubling time divided by ln 2.

which is an *ad hoc* and highly artificial assumption. The heterogeneity of the population would have to be such that there were as many components, as a result of a single mutation, as there are points of differing specific activity in Figure 1.

On the contrary, if the rate of aporepressor synthesis is inversely temperature-dependent, then each point in Figure 1 simply represents a different steady state level fixed by the constant rate of inactivation and a different rate of synthesis. An inverse temperature dependence in rate of synthesis is entirely plausible if an enzyme concerned with aporepressor formation has been rendered temperature-sensitive by the mutation. Garen and Echols[16] have suggested, on the basis of elegant genetic experiments, that the R2 regulator gene of strain K12 specifies the structure of an enzyme which converts an internal inducer of alkaline phosphatase synthesis into a repressor. Crosses of PR1 with appropriately marked donor strains confirm that the constitutive mutation in PR1 is at the R2 locus (Table 1).

It is likely that the synthesis of aporepressor represents a function distinct from its growth-dependent inactivation. Thus, the inactivation documented in Figure 6 probably reflects a metabolic turnover which occurs in repressible strains but which is masked by the high and invariant rate of synthesis. An initial e-fold increase in differential rate occurs in approximately 0.2 generations (Fig. 6), which suggests a time constant for aporepressor turnover of this order of magnitude. This estimate is rough, of course, since we have no independent information on the order of the reaction or on the relationship between aporepressor concentration and differential rate of enzyme synthesis.

TABLE 1

MAPPING OF PR1

| Recipient | Donor | Recombinant class screened | Colonies screened (no.) | Repressed colonies (no.) | Repressed colonies (%) |
|---|---|---|---|---|---|
| PR1LS | W 2252 | $Sm^rLeu^+$ | 571 | 14 | 2.5 |
| $Leu^-Mal^-Sm^rVal^r$ | $R1^+R2^+Leu^+Sm^sVal^sMal^+$ | | | | |
| | | $Mal^+Val^r$ | 44 | 3 | 7.0 |
| PR1LS | C 8 | $Sm^rLeu^+$ | 184 | 6 | 3.0 |
| | $R1^-R2^+Leu^+Sm^sVal^sMal^+$ | | | | |
| | | $Mal^+Val^r$ | 1697 | 111 | 6.5 |
| PR1LS | C 97 | $Sm^rLeu^+$ | 2584 | 4 | 0.15 |
| | $R1^+R2^-Leu^+Sm^sVal^sMal^+$ | | | | |
| | | $Mal^+Val^r$ | 994 | 1 | 0.1 |

The recipient strain PR1LS is a streptomycin-resistant leucine requiring derivative of PR1. Like all our strains of *E. coli* B, it is resistant to valine inhibition. The donor strains are derivatives of *Hfr Cavalli* which are sensitive to valine. C8 and C97 were generously supplied by Dr. Allan Garen. Log phase donors were shaken gently in Pennassay broth at 37° with an excess of recipients. After 120 min the cultures were diluted 20-fold in tryptone broth and cultivated at 37° for 120 min. They were then harvested, washed, and plated. Recombinants were selected on tris minimal-excess phosphate medium supplemented with 10 μg per ml thymine and 20 μg per ml methionine. For $Sm^rLeu^+$ recombinants the carbon source was 0.2% glucose, and streptomycin was present at 30 per ml. For $Mal^+Val^r$ recombinants the carbon source was 0.4% maltose, leucine was present at 20 μg per ml, and valine at 400 μg per ml. The plates were incubated at 37°. Alkaline phosphatase production was determined by spraying the recombinant colonies with p-nitrophenylphosphate at 15 mg/ml in 1 *M* tris, pH 8.0.

We suggested in the introduction that a steady state situation of this sort would frustrate physiological studies on enzyme repression because of the rapidity with which the aporepressor level would return to equilibrium after it had been disturbed. If our analysis is correct, PR1 should have the distinctive and useful property of returning more slowly to equilibrium, the higher the temperature. Experiments showing that this is precisely the case are reported elsewhere.[17] This property has enabled us to carry out inhibitor studies on PR1, under the hoped-for conditions of slow aporepressor synthesis, which provide circumstantial evidence as to the nature of the aporepressor.[18]

*Summary.*—The synthesis of alkaline phosphatase has been examined in a mutant of *E. coli* B in which repression by inorganic phosphate decreases with temperature. It is concluded that the temperature sensitivity lies in the rate of synthesis of an aporepressor which is normally metabolically unstable. The time constant for aporepressor turnover is estimated to be about 0.2 generations.

* This work was partially supported by grant NG-9546 from the National Institutes of Health.

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## *A MOLECULAR MECHANISM OF MITOMYCIN ACTION: LINKING OF COMPLEMENTARY DNA STRANDS*[*,†]

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*Communicated by Kenneth B. Raper, June 7, 1963*

The selective action of the antibiotic mitomycin C (MC)[1] on deoxyribonucleic acid (DNA),[2–6] together with its reported antineoplastic,[1, 7] mutagenic,[8] and phage-inducing[9] activities, has stimulated several investigations on the mechanism of its action. The preferential inhibition of bacterial DNA synthesis by MC, accompanied by progressive and extensive breakdown of the DNA, indicates that DNA is the principal target. However, the rapidity of MC-induced "death" seemed to be out of step with the relatively much slower process of DNA breakdown. This suggested that the effects hitherto observed might be secondary to an earlier action of the antibiotic on DNA. Such a primary lesion is described here and interpreted as *in vivo* MC-induced linking ("cross-linking") of the complementary strands of the DNA molecule.

*Materials and Methods.*—Samples of mitomycin C were kindly provided by Dr. J. Lein, Bristol Laboratories, Syracuse, N. Y., by Dr. R. B. Ross, Cancer Chemotherapy National Service Center, NIH, Bethesda, Md., and by the Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. The bacterial strains used included *Escherichia coli* strain B, *Sarcina lutea* strain ATCC-272, and the following *Bacillus subtilis* mutant lines:[10, 11] wild-type, the indole-requiring, the linked indole- and histidine-deficient ($I^-$, $H^-$), and a prototrophic derivative of the indole-requiring strain 168, the latter bearing in addition a marker (*mac-r*1) conferring resistance to the macrolide group of antibiotics. Bacteria which were growing exponentially in Difco's antibiotic medium 3 (Penassay broth) were exposed to MC under the conditions specified, and their survival determined by plating on nutrient agar. To terminate MC exposure the cells were chilled, washed twice with cold SSC (0.15 *M* NaCl + 0.015 *M* $Na_3$·citrate), and frozen with 100 μg lysozyme/ml. The procedures used for the isolation of protein- and RNA-free DNA and for the determination of its buoyant density and transforming activity were outlined earlier.[11] Thermal transition ("melting") curves were obtained with a recording thermospectrophotometer.[12] Thermal denaturation was carried out by exposing the DNA in 0.015 *M* NaCl + 0.0015 *M* trisodium citrate at pH 7.7 (DSC) to 100°C (or other specified temperatures) for 6 min followed by rapid cooling in an ice bath.[11]

*Results.*—The exposure of an exponentially growing culture of *B. subtilis* to an inhibitory concentration of MC resulted in very rapid cell death, colony-forming capacity dropping by several powers of ten in a matter of minutes (Fig. 1, Table 1). Native DNA extracted at this early period from MC-exposed cells and examined

TABLE 1

EFFECT OF MITOMYCIN C (MC) ON CELL SURVIVAL (%) AND TRANSFORMING ACTIVITY (% T.A.)*

| Exposure to MC (min) | MC concentration (μg/ml) | | | | |
|---|---|---|---|---|---|
| | 0.1 | 0.5 | 1.0 | 2.5 | 12.5 |
| 1 (*B. subtilis*) | — | — | 100 | — | 0.1 |
| 5 " | — | — | 100 | — | $4 \times 10^{-5}$ |
| 15 " | 100 | 10 | 0.8 | 0.6 | $10^{-6}$ |
| 15 (*E. coli*) | 5 | — | $10^{-2}$ | — | $2 \times 10^{-7}$ |
| 15 (% T.A.) | 100 | 54 | — | 45 | 21 |
| 30 (*B. subtilis*) | — | — | $4 \times 10^{-3}$ | — | $<10^{-8}$ |
| 60 " | — | — | $3 \times 10^{-4}$ | — | $<10^{-8}$ |
| 60 (% T.A.) | — | — | — | — | 6.7 |
| 120 (*B. subtilis*) | 100 | — | $3 \times 10^{-5}$ | — | $<10^{-8}$ |
| 300 " | 100 | — | $10^{-5}$ | — | — |

* Exponentially growing cells ($1–5 \times 10^8$ cells/ml, 37°C, Penassay broth) exposed to MC for indicated periods of time. Colony count determined after plating appropriate dilutions on nutrient agar. Transforming activity (indole$^+$) determined for RNA-free DNA extracted from separate aliquants of MC-treated cells.

by the technique of equilibrium density-gradient centrifugation was indistinguishable from DNA extracted from unexposed cells. Its transforming activity was only slightly diminished (Fig. 1, Table 1). However, when the DNA's from the control and MC-exposed cells were heat-denatured and rapidly cooled, a significant difference in the banding pattern in CsCl and $Cs_2SO_4$ gradients was revealed. Only a small fraction of the denatured DNA from MC-exposed cells (Figs. 2*B* and 3*B*) banded in the position normally occupied by the denatured normal (dN) DNA (Figs. 2*A* and 3*A*), while the main band formed at the density characteristic for the native (NN) or renatured state (Figs. 2*B* and 3*B*). Such behavior, by analogy with the effects of ultraviolet light[11, 13] and nitrous acid,[14] could be interpreted as resulting from thermostable linking of the complementary strands of the DNA molecules.

*Effects of formaldehyde (HCHO) and hydrodynamic shear on the denaturation process:* A scheme involving MC-induced linking of the complementary DNA strands poses two questions: (1) Do the linked strands fail to separate, even partially, when heated, or is the renaturation process very fast and efficient? (2) How frequent are the cross links? Firstly, when renaturation of the heat-treated MC-linked DNA was partially prevented by HCHO[11] (see Fig. 2*C*), no band was formed at the native DNA density, indicating that the apparent heat stability of MC-linked DNA can be attributed to its spontaneous renaturability in the absence of HCHO. Secondly, if the links are infrequent (one or only a few per molecule), shearing of

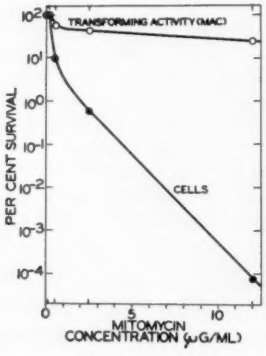


FIG. 1.—Survival of *B. subtilis* cells (●) (colony-forming ability) grown in Penassay broth and exposed for 15 min (37°C) to the indicated concentrations of mitomycin C (0, 0.1, 0.5, 2.5, 12 μg/ml), and of the specific transforming activity (○) (macrolide resistance marker) determined for deproteinized and RNA-free DNA extracted from the above cells. Other markers gave similar results (47, 74, and 48% survival at 2.5 μg MC/ml, and 16, 31, and 22% survival at 12.5 μg MC/ml for indole, histidine, and linked indole-histidine markers, respectively). Transforming activity for each marker was determined at DNA concentrations of 0.01, 0.1, 1, and 10 μg/ml. For each series of MC concentrations tested, the slopes of the resulting titration curves (DNA concentration versus number of transformant colonies) were essentially parallel.

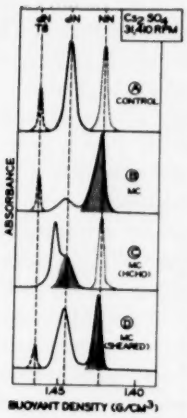


FIG. 2.—Microdensitometer tracings of photographs taken after 40 hr of $Cs_2SO_4$ equilibrium density-gradient centrifugation (31,410 rpm; 25°C) of DNA extracted from *B. subtilis* cells, never exposed to mitomycin (*A*), or grown for 15 min in the presence of 12.5 μg mitomycin (*B,C,D*), denatured (6 min 100°C; 0.02 *M* $Na^+$; pH = 7.8), and rapidly chilled at 0°C. In experiment *C*, 1% HCHO was present during denaturation, and 0.2% HCHO during centrifugation. The DNA used in experiment *D* was mechanically sheared prior to denaturation (forced 3 times through a gauge 25 needle (I.D. = 0.254 mm), using a spring-loaded constant rate syringe CR 700, Hamilton Company, Inc., Whittier, Calif., at a concentration of 15 μg/ml). The shaded areas roughly correspond to the mitomycin-linked DNA. The NN peaks (native *B. subtilis* DNA—dotted lines) and the dN-T6 peaks (denatured, rapidly cooled coliphage T6 DNA—broken lines) were obtained by repeating the centrifugation after addition, directly to the centrifuge cell, of 5 μl (1 μg) of the respective DNA's, serving as reference density markers. Buoyant densities: *B. subtilis* native DNA (NN)— 1.424, denatured (dN)—1.446. Sedimentation constants $S^0_{20,w}$ (in SSC) equal to 37 (*A*), 33 (*B,C*), and 19 (*D*), which correspond to mol. wts. of approximately 32, 23, and 5 × $10^6$, respectively (J. Eigner, personal communication). The proportion of denatured, nonlinked DNA, determined by comparing the areas under the peaks, increases upon shearing from 15% ($P_o$ = 0.15) (*B*) to 65% ($P_o$ = 0.65) (*D*).

the native cross-linked DNA should result in separation of the linked from the nonlinked DNA fragments and a decrease in the relative amount of native-like "molecules" upon subsequent denaturation (Fig. 7, third line), as revealed by density-gradient centrifugation. This prediction was verified in the experiment illustrated in Figures 2*B* and 2*D*. When shearing reduced the sedimentation constant of the DNA from $S^0_{20,w}$ = 30–40 to 15–20 S, the amount of cross-linked molecules decreased from 85 to 35 per cent.

*Dependence of the cross-linking on MC concentration, period of exposure, and temperature:* When exponentially growing *B. subtilis* cells were exposed to a concentration of 0.1 μg/ml MC at 37°C, multiplication of the cells was arrested, but there

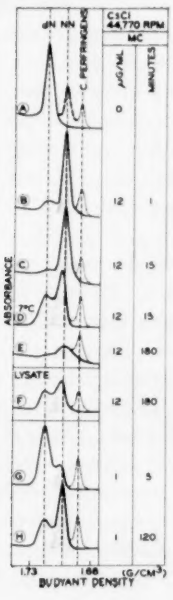


FIG. 3.—Microdensitometer tracings of photographs taken after 22 hr of CsCl equilibrium density-gradient centrifugation (44,770 rpm; 25°C) of DNA extracted from *B. subtilis* cells grown (nutrient broth) in the presence of 0 (*A*), 12 (*B–E*), or 1 (*G–H*) μg of mitomycin (MC) for the indicated periods of time (min). To prepare the DNA represented by tracing *F*, the cells were first frozen with lysozyme (100 μg/ml of SSC), thawed, and incubated for 30 min at 37°C, and the resulting lysate was exposed to 12 μg of mitomycin/ml. With the exception of tracing *D* (7°C), exposure to mitomycin was carried out at 37°C. All the DNA samples were denatured under the conditions outlined in the legend to Fig. 2. The broken (*B. subtilis*) and dotted (*Clostridium perfringens*) lines represent native, density-reference DNA's, added directly to the cells prior to repeating the centrifugation. Buoyant densities: *C. perfringens* native DNA—1.691 g/cm³; *B. subtilis* native DNA (NN)—1.703, denatured DNA (dN)—1.718 g/cm³. Frequencies of MC links: 1 link per mol. wt. of 10 × $10^6$ (*C*), 34 × $10^6$ (*D*), 135 × $10^6$ (*G*), 30 × $10^6$ (*H*).

was no observable reduction in viability during the 20-hr period of observation (Table 1). By the experimental criteria employed, DNA extracted from these cells behaved like normal DNA. The MC-imposed anomaly in the banding pattern of the denatured DNA became apparent at higher concentrations of MC. The relative amount of the DNA that resists denaturation (shaded areas) increased with increasing concentrations of MC (1–12 $\mu$g MC/ml; Figs. 3*G* and 3*B* or 3*C*), or increasing periods of exposure (5–120 min; Figs. 3*G* and 3*H*); it was also a function of the temperature (7° versus 37°C; Figs. 3*D* and 3*C*). For intermediate degrees of reaction with MC, the heated and rapidly cooled DNA comprised two distinct molecular classes, one of which had the buoyant density of denatured normal molecules, and the other (spontaneously renatured cross-linked molecules; shaded areas in Figs. 2 and 3) banding at a buoyant density slightly greater than that of the native normal molecules. On further reaction, the fraction banding in the renatured region increased until there was no detectable banding in the denatured region, unless hydrolytic or mechanical breakdown of the DNA intervened. Spreading of the bands (Fig. 3*E*) and a decrease in the alcohol precipitability of the DNA heralded the onset of its enzymatic destruction.

*Cross-linking in the absence of DNA and protein synthesis, and lack of in vitro interaction between purified DNA and MC:* The question of whether the changes induced by MC in DNA molecules *in vivo* are dependent on active synthesis of DNA or protein was examined by studying the effect of MC (12 $\mu$g/ml, 15 min, 37°C) under conditions of inhibition of these syntheses by 5-fluorodeoxyuridine (10 $\mu$g/ml added 5 min ahead of MC) and chloramphenicol (50 $\mu$g/ml added 15 min ahead of MC), respectively. Notwithstanding the previous exposure to and continued presence of these inhibitors, MC induced the molecular changes in the DNA to the same degree as in the absence of the inhibitors.

The implication that no concomitant DNA or protein synthesis is necessary for the MC-induced linking to occur was further strengthened by the observation that MC effectively cross-links purified *B. subtilis* DNA in the presence of either homologous or heterologous (*Sarcina lutea*) cell-free lysates prepared by lysozyme treatment and osmotic rupture (cf. Fig. 3*F* and legend). Nevertheless, MC failed to induce such changes in deproteinized DNA or DNA plus RNA extracts even after prolonged periods of *in vitro* exposure to 100 $\mu$g MC/ml (banding profile identical to that in Fig. 3*A*). Likewise, the transforming activity of purified DNA was unaffected by MC *in vitro*.

*Stability of the effect:* The continued presence of MC in the culture was not a requirement for persistence of the observed effects on the DNA. In fact, 2 hr incubation (37°C) of MC-treated (15 min, 12 $\mu$g MC/ml), washed cells in the absence of the antibiotic failed to reverse the MC effects.

*Generality of the effect:* In the light of recent reports[11, 15] that a fraction of the DNA molecules of *B. subtilis* normally fails to undergo strand separation on thermal denaturation, it was important to determine whether the observed effects of MC were peculiar to this bacterial species or could be extended to others. DNA extracted from *Escherichia coli* strain B cells exposed to MC under similar conditions behaved in a manner analogous to the *B. subtilis* DNA on heating and rapid cooling. A mutant strain of *E. coli*, partially resistant to MC, required 10 times higher MC concentrations to exhibit comparable bactericidal and DNA cross-linking effects.

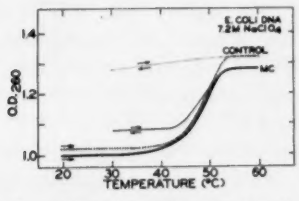


FIG. 4.—Thermal denaturation profiles in 7.2 *M* $NaClO_4$ obtained with the "recording thermospectrophotometer"[12] for DNA's isolated from *E. coli* cells grown in nutrient broth and exposed for 15 min to 0 (control—dotted lines) or 10 (MC—solid lines) μg mitomycin/ml. The first heating (0.5°C/min) is indicated by the thicker lines. Absorbance changes ($O.D._{260}$) during subsequent cooling (5°C/min) and heating (0.5°C/min) cycles, which were carried out in the thermospectrophotometer cuvette compartment, are represented by the thinner lines. The midpoint transition values, $t_m$ ("melting" temperature), correspond to 48.5°C (control) and 48.0°C (mitomycin-linked DNA).

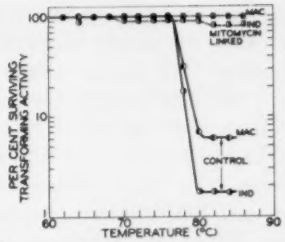


FIG. 5.—Thermal inactivation of transforming DNA extracted from *B. subtilis* cells grown in nutrient broth and exposed for 15 min (37°C) to 0 (solid lines) or to 12 (dotted lines) μg of mitomycin/ml. Each point corresponds to an assay performed on an 0.03 ml sample of DNA, dissolved in DSC (0.015 *M* NaCl, 0.0015 *M* $Na_3$-citrate, pH = 7.8) at 10 μg DNA/ml, sealed in a glass capillary, heated for 10 min at the indicated temperature, and rapidly chilled in ice water (0°C). Transforming activity was assayed for indole independence (IND) (minimal agar) and for resistance to the macrolide group of antibiotics (MAC) (0.5 μg erythromycin/ml), by plating 0.05 ml of a transforming mixture containing 0.002 μg of DNA and approximately $2 \times 10^7$ recipient cells.

In preliminary studies, we have also observed similar effects of MC on the DNA of cultured human cells and of replicating coliphage T4 or *B. subtilis* phage PBS 2, although 5–10 times higher MC concentrations were required.

*The thermal transition characteristics of "MC-linked" DNA:* The changes observed in the absorbance of *E. coli* DNA, both normal and MC-linked, slowly heated in 7.2 *M* $NaClO_4$,[12, 14] indicate that most of the hydrogen bonds "melted out" (Fig. 4, heavy lines). Differences were observed only in subsequent cooling and heating cycles, which were carried out in the thermospectrophotometer cuvette compartment. The temperature-absorbance profiles indicate that a high proportion of the MC-linked DNA returns to the native-like (hypochromic) state (Fig. 4, thin solid line) unlike the normal DNA (thin dotted line). Analogous results were obtained with *B. subtilis* DNA.

*Transforming activity of "MC-linked" DNA:* While MC-induced loss of cell viability and DNA cross-linking are rapid processes, the transforming activity of the DNA, tested for three different characters, is only gradually lost (Fig. 1). Even when cross-linking was quite extensive (Figs. 2*B* and 3*B*), as much as 20 per cent of the original transforming activity was still retained by the molecules. Under these conditions, the ability of two genetically linked markers ($I^+$, $H^+$) to be cotransferred was also retained, suggesting that the region concerned had not yet suffered any damage. Furthermore, the transforming activity of MC-linked DNA, unlike the activity of normal DNA, was *not* critically and abruptly destroyed over a narrow temperature range (Fig. 5). This remarkable heat stability of DNA isolated from MC-treated cells indicates that its residual transforming activity is primarily associated with the cross-linked DNA molecules.

On prolonged exposure to MC (over 2 hr), the ability of the $I^+H^+$ region to be cotransferred to the doubly deficient strain fell more rapidly than that of the component markers. A similar genetic dissociation of associated markers under the influence of MC has recently been reported by Yuki[16] in the *Escherichia coli* K12 system. Prolonged exposure to MC resulted in eventual loss of all detectable transforming activity and recoverability of polymerized DNA.

*Discussion.*—The most obvious interpretation of the foregoing results is the formation of rare links between complementary DNA strands by metabolically activated MC. The high chemical reactivity of this antibiotic could be inferred from its structure (Fig. 6): aziridine ring 1, 2, 1a, methylurethane at position 9, quinone structure 5, 8, and substitutions at positions 7 and 9a are all highly reactive groups. The polycyclic structure might favor intercalation of this compound into DNA,[18] as a step prior to selective cross-linking. The density shift in the presence of HCHO (Fig. 2*C*) and the increase in absorbance of the MC-linked DNA (Fig. 4, solid heavy line) attest to almost complete collapse of the hydrogen-bonded structure during thermal denaturation, as schematically indicated in Figure 7 (column *B*), although the MC-induced link does not permit complete separation of the complementary strands. This link, maintaining the original alignment, permits very rapid return of the DNA molecule to the original native, hydrogen-bonded structure, a process kinetically improbable for denatured normal DNA (column *C*). Assuming a Poisson's distribution of the links, and further, that one of these links is sufficient to permit the spontaneous renaturation of the molecule, it is possible to make a very rough estimate of the average number of cross-links per molecule, $m = \ln (1/P_0)$ where $P_0$ is the proportion of the molecules unlinked. Based on the numerical data derived from Figures 2*B* and 2*C*, the average frequency of MC-induced links corresponds to one per 20,000 nucleotide pairs, i. e., per mol. wt. $12 \times 10^6$ (1.8 links (*B*) per mol. wt. $23 \times 10^6$, or 0.43 link (*D*) per mol. wt. $5 \times 10^6$). Similar estimates for various conditions of MC treatment are listed in the legend of Figure 3.

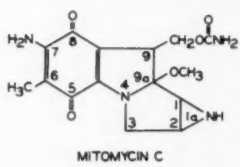


FIG. 6.—Structure of mitomycin C according to Webb *et al.*[17]

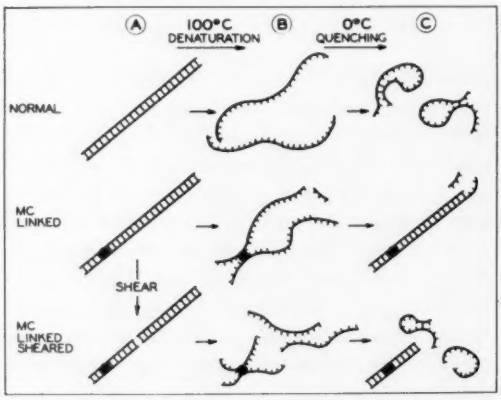


FIG. 7.—Molecular events following the denaturation (*A* to *B*) and rapid cooling (*B* to *C*) of normal and MC-linked DNA, the latter before and after subjection to hydrodynamic shear. The possibility of hydrolytic breakage of the phosphate ester bonds and its consequences are indicated for MC-linked DNA (middle row).

From our present findings and those of others,[2-6] it is suggested that the "cross-links" imposed by MC (approximately 50 per bacterial DNA complement for 5 min exposure to 1 $\mu$g MC/ml; Fig. 3*F*) (1) interfere with DNA replication; (2) rapidly lead to cell death;[19] (3) nevertheless have little effect on the ability of the DNA to transform for a given marker[20] or direct the synthesis of one or a few proteins,[21] since these two activities reflect a function of a relatively small fraction of the total DNA complement. The reported partial resistance of viral DNA replication to the action of MC[3, 9, 22] remains to be explained, but may reflect the relatively smaller size of the viral DNA molecules and/or their fragmentation[23] during the replication process.

The observation that some natural bacterial and viral DNA's appear to be cross-linked,[11, 15, 24] together with the results here obtained with mitomycin, which is a natural product, suggests some biological role for this cross-linking process. If reversible, this process would permit turning on or off DNA synthesis, and thus would assume a regulatory significance. Although the determination of the exact chemical nature of the cross-links might be difficult because of their low frequency, the presently acquired knowledge of the mechanism of MC action permits application of this antibiotic in a variety of experiments, both with intact cells[25] and transforming DNA.

*Summary.*—Exposure of *Bacillus subtilis* or *Escherichia coli* cells to inhibitory concentrations of mitomycin C for periods of 1–60 min results in covalent linking of the complementary DNA strands. This cross-linked DNA denatures upon thermal treatment, but renatures spontaneously even with rapid chilling, as revealed by its return to the native-like buoyant density in CsCl or $Cs_2SO_4$ gradients and by the reversibility of the optical density changes during heating and cooling cycles. Hydrodynamic shearing of the cross-linked DNA permits separation of the linked and nonlinked regions.

Only $10^{-4}$ per cent of *B. subtilis* cells survive 15 min exposure to 12 $\mu$g of mitomycin per ml. Transforming DNA isolated from cells so treated (one cross-link per mol. wt. of 10–12 million) retains 20–30 per cent of its activity, which becomes resistant to thermal denaturation. Higher concentrations of mitomycin are required to effect a comparable degree of cross-linking of vegetative $T_4$ or PBS 2 phage DNA and the DNA of human cells or mitomycin-resistant bacterial mutants. Mitomycin has no *in vitro* effect on purified DNA unless a cell extract is added.

We are indebted to Dr. Elizabeth H. Szybalska for her editorial help, to Dr. I. Takahashi for the *B. subtilis* strain carrying the *mac-r*1 marker and for the phage PBS 2, to Dr. H. Gottschling for the phage T4(N24), to Mrs. Z. Opara-Kubinska for her counsel with the transformation experiments, and to Mr. Larry Fenton, Mr. Hans Kvinlaug, and Miss Gwen Nettles for their able technical assistance.

* These studies were supported by grant G-18165 from the National Science Foundation and by grant CY-5215 from the National Cancer Institute, USPHS. Contribution No. 559 from the Microbiology Research Institute, Canada Department of Agriculture, Ottawa.

† The following abbreviations are used in this paper: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; RNase, ribonuclease; MC, mitomycin C; UV, ultraviolet light.

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# *INACTIVATION OF BACTERIOPHAGE BY CHLOROFORM AND X IRRADIATION**

By Irene M. Cotton and Lloyd S. Lockingen

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*Communicated by Wilson S. Stone, June 25, 1963*

Chloroform is sometimes added to stock cultures of bacteriophage to prevent microbial contamination.[1] During the course of experiments on the effect of nitric oxide on X-ray inactivation of bacteriophage it was noted that phage stocks which had been preserved with chloroform always showed greater sensitivity to X irradiation than those not so preserved. The purpose of this communication is to report the results of preliminary investigations into the relationship between chloroform and X-ray inactivation of T2r phage and to describe conditions under which the lethal effect as judged by surviving fraction following a given dose of X rays can be increased by as much as 500,000 times in the presence of small amounts of chloroform.

*Materials and Methods.*—Preparation and purification of phage stocks and assay procedures have been described.[2] Spectroquality reagent grade chloroform was a product of Matheson, Coleman, and Bell. The irradiations were carried out with a Quadrocondex constant potential X-ray machine operated at 220 kv and 15 ma. No filtration was used. The relative dose rate was determined before and after the experiments with a Victoreen Radocon. Since this instrument was calibrated using filters, the doses given in the tables and figures are low by a small unknown factor, and they should be considered as relative rather than absolute. Ultraviolet light was delivered by a model 30600 Hanovia Quartz Lamp through filters 6540 and 6541 which limited transmission to the 2,500–3,700 Å range.

Bacteriophage were diluted into M-9 salts solution (no glucose) and treated with chloroform. Since chloroform is relatively insoluble in water, the highest concentration used was 4 $\mu$l/ml of phage suspension. The procedure for treating the phage was to deliver 20 $\mu$l of chloroform into 5 ml of phage suspension in a Pyrex screw cap tube. The mixture was then manually shaken until there was no visible evidence of chloroform droplets. It was then allowed to equilibrate for 10 min, after which time 4 ml of the treated suspension was placed in a 10 ml Pyrex Griffen beaker and irradiated. It was found that beakers which had been repeatedly irradiated were unsatisfactory, since erratic survival curves resulted. Discolored beakers were discarded.

*Results.—Early experiments:* The earliest experiments (Figs. 1 and 2) clearly showed that the presence of chloroform during irradiation significantly reduced the surviving population of T2r bacteriophage. The resulting survival curves were not, however, quantitatively reproducible from day to day and were not exponential. The system responded to changes in the method of adding chloroform to the phage and in the time of exposure to chloroform before irradiation. The procedure previously described was instituted to minimize these effects, and it did result in fair quantitative reproducibility. Since the most interesting part of the survival curve seemed to be at total doses of 5 Kr, the relative dose rate was shifted from 1,000 r/min to 250 r/min to define the curve more adequately.

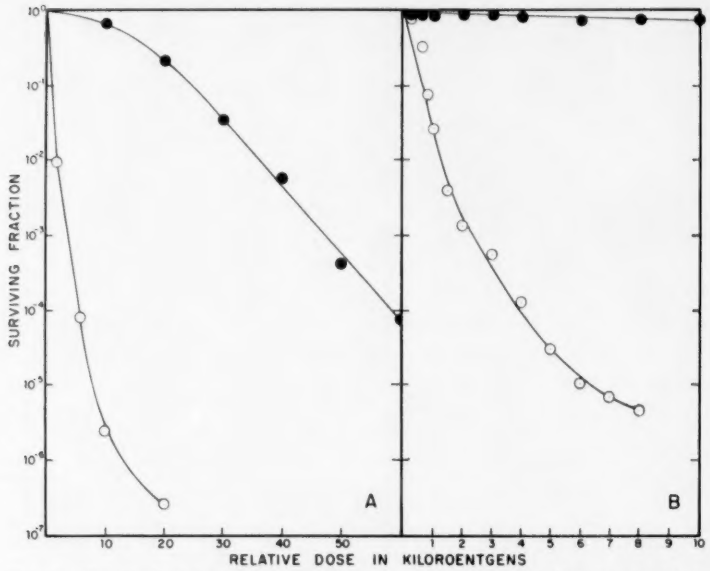


FIG. 1.—Survival of bacteriophage after X irradiation. ⊙ chloroform added, ● no chloroform. *A*, 1000 r/min., *B*, 250 r/min.

Because the curves are not exponential and because survival in the control experiments is high, there is no convenient way of expressing a relationship between the two curves except in terms of the surviving fraction at some fixed total dose. For example, a rough estimate of the $D_{50}$ from Figure 3 is 100 r for the chloroform-treated culture and 15,000 r for the broth-chloroform control culture. The surviving fractions at 4 Kr are 0.0000013 and 0.90, respectively, a ratio of approximately 700,000.

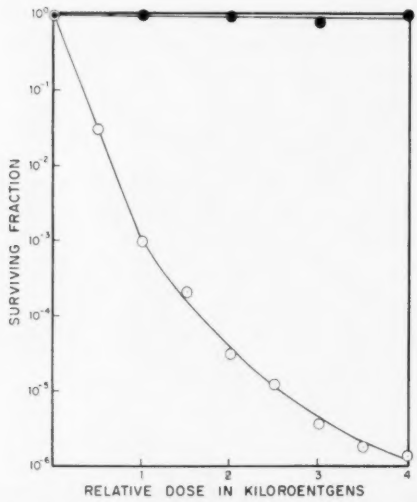


FIG. 2.—Broth reversal of the action of chloroform and X irradiation of bacteriophage. ● survival of phage suspended in broth and chloroform. ⊙ survival of phage in chloroform. 250 r/min.

*Pretreatment of bacteriophage with chloroform:* It was important to know whether or not chloroform interacted with the bacteriophage in such a way as to sensitize the phage to subsequent irradiation. Suspensions of phage were pretreated with 0.4 per cent chloroform and then were divided into two parts, one of which was vigorously bubbled with helium for three min prior to irradiation. Table 1 presents data from two such experiments which demonstrate that the chloroform does not irreversibly sensitize the phage to subsequent irradiation.

*Broth reversal of chloroform effect:* It was then necessary to determine whether or not the chloroform effect was similar to the indirect effect of irradiation in that chemical agents were produced in the solvent. The first tests utilized nutrient broth, one of the classic agents in bacteriophage experiments, for the suppression of the indirect effect. The results are shown in Figure 2. The addition of only 5 per cent V/V of broth to the M-9 salts-chloroform-phage system, which was a final concentration of 0.09 per cent broth solids, completely blocked the effect of the chloroform.

*Aftereffect:* If the effect of chloroform was due to the production of toxic products in the solvent, then the study of phage survival in M-9 salts and chloroform after the cessation of irradiation should show the existence of such products. Parallel suspensions of phage in M-9 salts and in M-9 salts plus chloroform were irradiated at a dose rate of 250 r/min for eight min. At the termination of irradiation these suspensions were sampled periodically for 40 min. Figure 3 shows the combined results of two such parallel experiments and indicates a greater progressive loss of bacteriophage treated with chloroform than with M-9 salts alone. At 40 min after irradiation about 85 per cent of the bacteriophage treated with chloroform were inactivated as compared with 15 per cent in the case of the M-9 salts controls.

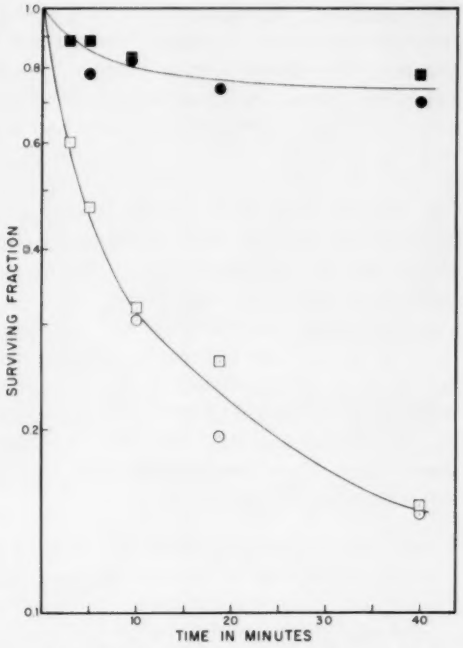


FIG. 3.—Survival of phage after cessation of radiation. ⊙, [·] phage irradiated in chloroform. ●, ■, phage irradiated in M-9 salts only. Duplicate experiments shown.

*Indirect effect:* Other tests have been made to reveal the indirect effect in the

TABLE 1

EFFECT OF BUBBLING WITH HELIUM ON THE EFFECT OF CHLOROFORM AND X IRRADIATION

| | Titers* Exp. 1 | | Titers* Exp. 2 | |
|---|---|---|---|---|
| Dose in roentgens | Not bubbled | Helium bubbled | Not bubbled | Helium bubbled |
| Starting | 2.31 | — | 1.77 | — |
| After chloroform | 2.02 | 2.02 | 1.20 | 1.20 |
| After bubbling | — | 1.71 | — | 1.09 |
| 250 | — | — | 0.85 | 1.00 |
| 500 | — | — | 0.18 | 1.01 |
| 1,000 | 0.038 | 1.52 | 0.16 | 0.94 |
| 1,500 | — | — | 0.0026 | 0.90 |
| 2,000 | 0.0020 | 1.50 | 0.00075 | 0.90 |
| 2,500 | — | — | 0.00021 | 0.87 |
| 3,000 | 0.000082 | 1.45 | — | 0.87 |
| 4,000 | 0.000018 | 1.29 | 0.000017 | 0.77 |

* $\times 10^9$; for description see text.

following way. A suspension of M-9 salts and 0.4 per cent chloroform was prepared as previously described, but without the addition of bacteriophage. In a separate tube a suspension of phage in M-9 salts was prepared without chloroform. After the 10-min equilibration period, the two suspensions were divided, and aliquots of each were irradiated simultaneously in separate containers at 1,000 r/min for 30 min. At the termination of irradiation four mixtures were made as follows: (I) nonirradiated M-9 salts-chloroform and nonirradiated phage; (II) nonirradiated M-9 salts-chloroform and irradiated phage; (III) irradiated M-9 salts-chloroform and nonirradiated phage; and (IV) irradiated M-9 salts-chloroform and irradiated phage. Sampling began as soon as the mixtures were made and continued for 40–60 min. In calculating the surviving fraction, the first sample (i.e, directly after mixing) was used as the reference number. Table 2 summarizes the results of these experiments. There is a significant inactivation of nonirradiated phage when placed in the irradiated salts-chloroform solution (III), the reduction being about 90 per cent. When irradiated phage are placed in irradiated salts-chloroform solution (IV), there is a significantly greater inactivation of the bacteriophage than with nonirradiated phage in irradiated M-9 salts-chloroform (III).

TABLE 2

INDIRECT EFFECT

| | Surviving fraction $\times 10^3$ | | | |
|---|---|---|---|---|
| M-9 salts chloroform-irradiated | No | No | Yes | Yes |
| Phage-irradiated | No | Yes | No | Yes |
| Exp. 1 (after 60 min) | 0.95 | 0.55 | 0.046 | 0.00084 |
| Exp. 2 (after 40 min) | — | 0.55 | 0.054 | 0.023 |
| Exp. 3 (after 60 min) | 0.23 | — | 0.016 | <0.00015 |

*Ultraviolet light:* Survival of bacteriophage treated with ultraviolet light at wavelengths between 2,500 and 3,700 Å were exponential and were not influenced in any demonstable way by the presence of 0.4 per cent chloroform. Figure 4 shows the results of a typical experiment.

*Discussion.*—In 1941, Luria and Exner[3] demonstrated that bacteriophages irradiated in minimal salts solution were subject to greater damage by X irradiation than were phages irradiated in nutrient broth. Since that time several substances have been found which are of a protective nature to the phages, but relatively few have been studied which produce an increased sensitivity to radiation.

The present experiments show that interactions exist among irradiated and nonirradiated chloroform solutions in buffer and irradiated and nonirradiated phage particles. In some instances surviving fractions to a given dose are more than $10^5$ times lower than those observed under other circumstances. Whether these relations are additive or synergistic in nature cannot be said now, since this judgment will depend upon recognition of rates of inactivation of the phage under the different experimental circumstances.

It is clear, however, that the inactivating mechanism does require both chloroform and possibly ionizing radiation. That chloroform must be present during irradiation is indicated since (1) bubbling for 3 min with helium before irradiation completely suppressed the effect; (2) adding chloroform to nonirradiated phage resulted in very small losses; (3) a greater inactivation of phage was found in the

aftereffect experiments when chloroform was present during the irradiation; and (4) at least a 10-fold inactivation was found in the indirect effect experiments when irradiated M-9 salts-chloroform was added to nonirradiated phage.

The kind and quality of radiation required to produce the effect was not defined by these experiments, except that the effect was produced by X irradiation and not by ultraviolet irradiation at wavelengths between 2,500 and 3,700 Å. In this respect it is significant to note that the first experiments with ultraviolet irradiation of the medium in the indirect production of mutations required wavelengths below 2,000 Å and that the longer wavelengths, which do not activate water, were ineffective.[4] It remains for future experiments to determine whether shorter wavelengths of ultraviolet will produce an effect with chloroform

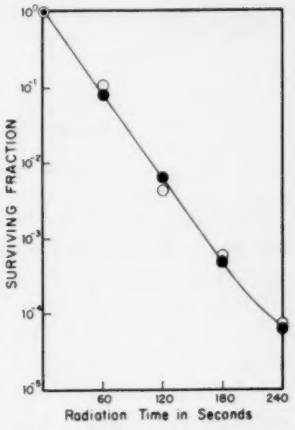


FIG. 4.—Survival of bacteriophage after ultraviolet irradiation. ⊙ chloroform added. ● no chloroform.

The nature of the chloroform effect is not understood, but it is strongly indicated that there exists in this sytem a phenomenon closely related to the indirect effect. It is possible, but it has not been shown, that radicals are the toxic products formed in the solvent.[5] That such toxic products were formed in the solvent has been clearly demonstrated by (1) reversal of the effect by small concentrations of nutrient broth (which also reverses the indirect effect), (2) by an increase in phage inactivation after the cessation of irradiation when chloroform was present during irradiation, and (3) by the inactivation of phage after being placed in M-9 salts-chloroform solutions which had been irradiated before introduction of the phage.

These results lead to the conclusion that chloroform is active directly or catalytically in the production of toxic products in the solvent.

The authors wish to thank Professor Wilson S. Stone for his support and inspirational leadership, and acknowledge gratefully the technical assistance of Claire LeBreton, Kenneth N. Bush, Jeff Roberts, Barry Schwarz, and Sharon Rae.

* This investigation was supported (in part) by USPHS research grant (GM-06492) and training grant (2G-337) from the National Institutes of Health, U.S. Public Health Service, by contract AT-(40-1)-2952 with the Atomic Energy Commission, and by a grant from the Robert A. Welch Foundation.

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# *SPECTROSCOPIC INVESTIGATION OF THE REACTION OF SPERM WHALE MYOGLOBIN WITH ZINC**

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*Communicated by Theodore T. Puck, June 17, 1963*

The relationship of macromolecular structure to biological activity will be understood in its totality only when complete structural determinations of representative macromolecules have been made. But even then, as now, a variety of physico- and biochemical methods will necessarily be brought to bear on such problems as the nature of the forces which determine and maintain structural integrity and the effect of distribution, spatial arrangement, and state of the various reactive groups in a protein molecule upon structure and biological activity. These experiments are naturally of interest for molecular genetics and the biosynthesis of proteins because they provide, among other things, information concerning the extent to which the amino acid sequence of a polypeptide chain determines *per se* its state of folding in a globular protein. Explanation, for example, of how such a slight change in primary structure as replacement of a single glutamic acid residue in hemoglobin A by valine in hemoglobin S can so profoundly modify the physiological activity of the protein, will be a major advance in our understanding of life processes. One approach to these problems is the study of complex formation between metallic cations and proteins. In fact, because of their widespread occurrence and important biological activities, metal-protein complexes constitute in themselves a problem of considerable interest.[1] Provocative problems in this area are concerned with the nature of the heme-heme interaction in hemoglobin and the possible involvement of sulfhydryl groups in its mechanism; unmasking of metal binding sites on some protein molecules by denaturation or, as in the case of the interaction of $Zn^{++}$ with serum albumin, simply by raising the temperature from 0° to 37°C; stabilization of some proteins to enzymatic digestion or heat by metal ions; structure and mechanisms of action of metalloenzymes; role of $Mg^{++}$ in the structural stability of ribosomes; and binding of metal ions into chelate structures as in the case of complexes of hemoglobin with $Hg^{++}$, $(Fe^{+++})_2$-conalbumin, and $Zn^{++}$-insulin.

The specific and reversible combination of $Zn^{++}$ with a variety of proteins often results in insoluble $Zn^{++}$-protein complexes. This fact underlies a system of fractionation of the plasma proteins[2] and a method of separating fetuin from the other proteins of fetal serum.[3] It has now been demonstrated spectroscopically that combination of $Zn^{++}$ with myoglobin causes reversible alterations in macromolecular conformation. Myoglobin is an ideal protein for such investigations since its exact three-dimensional structure has been largely established by the crystallographic X-ray analyses of Kendrew and co-workers,[4] thereby providing the fundamental information required for a detailed description of the mechanisms of its reactions. The realization that binding of $Zn^{++}$ by myoglobin mediates conformational changes has important implications, not only for methods of fractionation of biological materials, but also for the mechanisms of biochemical reactions such as the metal-activation and poisoning of enzymes.

*Experimental.*—Solutions of ferri(met)myoglobin (Mann's crystalline material, dialyzed free of salt and lyophilized), containing $9 \times 10^{-3}$ *M* zinc acetate and 0.04 *M* KCl, pH 6.4, were incubated for $3\frac{1}{2}$ hr at 28.4°C or 10 min at 46°. In control experiments, sodium acetate was substituted for zinc acetate. Reaction of the protein with $Zn^{++}$, as revealed spectroscopically, was reversed to yield renatured ferrimyoglobin by one of the following three methods: (*a*) adjustment of the pH of the reaction mixture to a value of 5.2 with acetic acid followed by incubation at room temperature for 7 hr; (*b*) dialysis of the reaction mixture for 5 hr versus 0.02 *M* acetate buffer pH 5.3; or (*c*) at pH 6.4 by addition of one volume of 0.05 *M* ethylenediamine tetraacetate (EDTA). Ferromyoglobin were prepared by reduction of native, $Zn^{++}$-reacted, or EDTA-renatured ferrimyoglobin with about $2 \times 10^{-4}$ *M* sodium dithionite. For determination of the differential absorption spectra, reaction mixtures containing varying concentrations of protein were prepared at 1°C, at which temperature the reaction leading to spectroscopic changes does not occur. A portion of each mixture was incubated for 10 min at 46°, while another portion was maintained at 1°C. The difference spectrum of the unreacted portion versus the reacted portion was measured in a Cary Model 14 spectrophotometer. In kinetic experiments at constant $Zn^{++}$ concentration the ionic strength was adjusted with KCl. Plots of the logarithm of the initial logarithm rate of reaction versus the square root of ionic strength were extrapolated to zero ionic strength.

*Results.*—Reaction of sperm whale ferrimyoglobin with $Zn^{++}$ at pH 6.4 results in major spectral changes. As shown in Figure 1, there is a marked reduction in the Soret band intensity of the protein accompanied by a shift of the band from an absorption maximum at 408 mμ to one at 390 mμ. Whereas the former band is quite sharp, the latter is diffuse. These spectral changes can be reversed either by adjusting the pH of the reaction mixture to pH 5.2, as illustrated in Figure 1, or by sequestering the $Zn^{++}$ at pH 6.4 with EDTA or citrate. The changes induced in Soret band absorption by reaction with $Zn^{++}$ are similar to, but not identical with, those accompanying acid denaturation.[5] The major difference is in the wavelength of maximum absorption which occurs at 390 mμ with $Zn^{++}$-reacted myoglobin and at 370 mμ with the acid-denatured protein. In a paper which appeared during preparation of our report, Breslow and Gurd[6] describe changes in the Soret band of myoglobin brought about by reaction with $Cu^{++}$ which are very similar to those described herein for $Zn^{++}$.

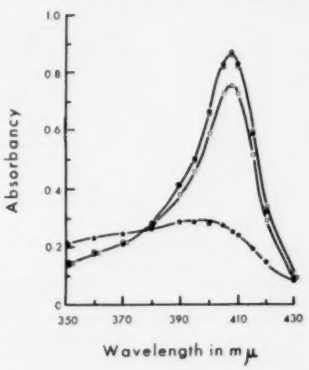


FIG. 1.—Soret band absorption spectra of ferrimyoglobin, 0.01 gm/100 ml: ○, native protein; ★, $Zn^{++}$-reacted protein; ●, renatured protein obtained by dialysis of $Zn^{++}$-reacted protein versus acetate buffer, pH 5.3; □, renatured protein obtained by adjusting pH of solution of $Zn^{++}$-reacted protein from a value of 6.4 to 5.2.

Not only is the Soret band absorption of myoglobin altered by reaction with $Zn^{++}$, but also the absorption in the spectral region between 450 and 700 mμ. The spectra of native ferri-, ferro-, and oxygenated ferromyoglobin in this region are presented in Figure 2*A*, and those of the $Zn^{++}$-reacted ferrimyoglobin and its ferro- and oxygenated ferro-derivatives in Figure 2*B*. While all three spectra are markedly altered by reaction of the protein with $Zn^{++}$, the change is most striking in the case of the oxygenated ferro-derivative, the double-banded structure characteristic of oxymyoglobin being virtually absent. It would thus appear that, rather than forming a relatively stable oxygen to iron band with the ferroprotein derived from $Zn^{++}$-reacted ferrimyoglobin, the oxygen simply oxidizes the iron to its ferric state.

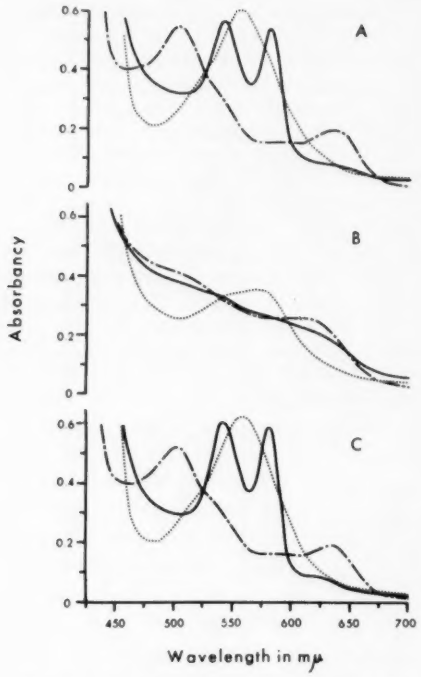


FIG. 2.—Absorption spectra of ferrimyoglobin, ——; ferromyoglobin, - - - - ; oxygenated ferromyoglobin, ——; *A*, native protein; *B*, $Zn^{++}$-reacted protein; *C*, EDTA-renatured protein. In each case, the ferriprotein was reduced with dithionite to the corresponding ferroprotein which was subsequently oxygenated. Protein concentration, 0.1 gm/100 ml.

It was found that, as in the case of Soret absorption, the changes in the 450–700 mμ spectrum of ferrimyoglobin due to reaction with $Zn^{++}$ are similar to, but not identical with, those brought about by acid denaturation. Whereas both processes nearly obliterate the 500 mμ band, acid denaturation does not appreciably affect the 635 mμ band. As shown in Figure 2*C*, the spectral changes incurred in ferrimyoglobin by reaction with $Zn^{++}$ are completely reversed by sequestering the metal ion in the reaction mixture with EDTA. Also, both the ferromyoglobin obtained from EDTA-renatured ferrimyoglobin by dithionite reduction and the subsequently derived oxymyoglobin show spectra identical with those of the corresponding proteins prepared from ferrimyoglobin never exposed to $Zn^{++}$. Furthermore, the renatured ferrimyoglobin can be readily crystallized in the same crystal habit as myoglobin never exposed to $Zn^{++}$.

The kinetics of reaction of ferrimyoglobin with $Zn^{++}$ were investigated by following the time-course of suppression of the Soret band intensity. The initial rate of reaction is first-order in both protein and $Zn^{++}$ and decreases by a factor of about 60 when the pH is lowered from 6.4 to 5.4. The reaction is very sensitive to temperature and has an activation energy of 46 kcal $mole^{-1}$ and an entropy of activation of 87 eu, values typical of protein denaturation. These results suggest that the rate-limiting step in the suppression of Soret band intensity involves the mutual binding of a single $Zn^{++}$ by two ionizable groups, presumably imidazole, on the protein molecule with concomitant, reversible, macromolecular conformational changes.

Supporting evidence for conformational changes was sought in difference ultraviolet absorption spectra. Glazer and Smith[7] discovered that the difference spectra of native versus denatured proteins show a prominent peak in the region of 230 mμ, which may arise largely from a change in peptide-bond absorptivity concomitant with a conformational change.[7,8] As illustrated in Figure 3, acid-denatured myoglobin, which is known to be extensively unfolded,[9] shows a difference peak at 229 mμ with a $\Delta\epsilon_M$ value of 26,400. Likewise, $Zn^{++}$-reacted myoglobin shows a difference peak at 228 mμ (Fig. 4) with a value of 16,700 for $\Delta\epsilon_M$ at low protein concentrations. Despite the complication of a curious discontinuity[10] in the nature

of the spectra and the plot of differential absorbancy versus protein concentration (Fig. 5), the conclusion seems justified that $Zn^{++}$-reacted myoglobin is conformationally quite different from the unreacted protein.

The recent paper of Breslow and Gurd,[6] referred to previously, also presents evidence for a reversible conformational change accompanying binding of $Cu^{++}$ or $Zn^{++}$ by myoglobin and for participation of imidazole groups in the metal ion binding.

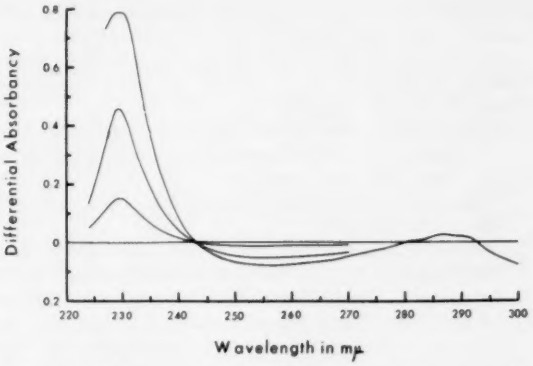


FIG. 3.—Difference absorption spectra of native versus acid-denatured ferrimyoglobin. Reading from bottom to top at 230 mμ, the three spectra correspond to protein concentrations of 0.01, 0.03, and 0.05 gm/100 ml. Native protein at pH 6; denatured, pH 3. Light path, 1.0 cm.

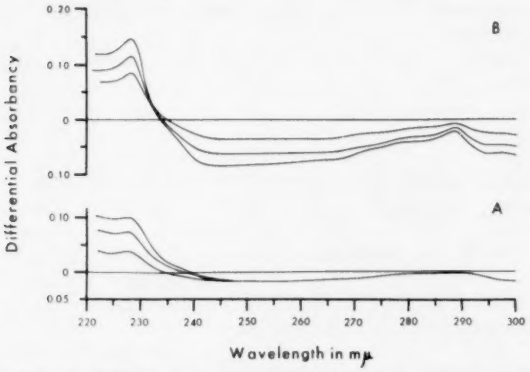


FIG. 4.—Difference absorption spectra of unreacted versus $Zn^{++}$-reacted ferrimyoglobin. Reading from bottom to top at 228 mμ, the spectra correspond to protein concentrations of: *A*, 0.005, 0.0075, and 0.01 gm/100 ml; *B*, 0.015, 0.0225, and 0.03 gm/100 ml. Light path 1.0 cm.

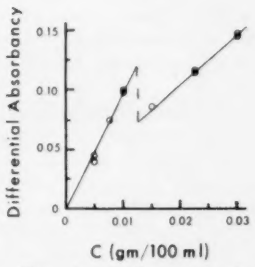


FIG. 5.—Differential absorbancy at 228 mμ for unreacted versus $Zn^{++}$-reacted ferrimyoglobin plotted against protein concentration. See reference 10 for discussion of discontinuity.

Finally, studies in progress in our laboratory show that, as in the case of myoglobin, combination of $Zn^{++}$ with hemoglobin causes major and reversible alterations in Soret band absorption.

*Summary.*—Combination of $Zn^{++}$ with myoglobin causes large and reversible changes in the ultraviolet and visible absorption spectra of the protein. The rate-limiting step in suppression of Soret band intensity appears to involve the mutual binding of a single $Zn^{++}$ by two ionizable groups on the protein molecule with concomitant, reversible macromolecular conformational changes.

* Contribution No. 209 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver, Colorado. This work was supported in part by research grant AI-01482-12 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, U.S. Public Health Service.

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[10] Although $Zn^{++}$-reacted myoglobin is aggregated as revealed by a sedimentation constant of about 4S as compared with 2S for unreacted protein, aggregation *per se* does not seem to account for the discontinuity. Within experimental error, the sedimentation constant is the same at a protein concentration of 0.03 as at 0.0075 gm/100 ml. In passing, it should be noted that, when reaction with $Zn^{++}$ is reversed, the sedimentation constant returns to its native value.

The apparent departure from Beer's law might simply be an instrumental artifact associated with the high absolute absorbances encountered at the higher protein concentrations, e.g., 1.7 at 228 mμ and greater than 2 at 225 mμ for a concentration of 0.02 gm/100 ml. Under these conditions stray light effects, which can act to lower and shift peak intensites,[11] could conceivably result in nonlinear dependency of differential absorbancy upon concentration.

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## *NONRECIPROCAL EVENTS AT THE GARNET LOCUS IN DROSOPHILA MELANOGASTER**

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*Communicated by M. Demerec, June 26, 1963*

Recent results in the genetics of higher organisms, particularly Drosophila, have suggested that tests involving two different mutant alleles of the same gene may yield wild-type products without apparent recombination. The first example in Drosophila involved the reddish mutant in *D. virilis*, [1, 2] but more recently the garnet (*g*) locus of *D. melanogaster* has similarly given such "unusual" results.[3, 4] The difficulty in interpreting these experiments has been the ambiguity as to whether the wild-type products were the results of closely positioned multiple crossovers (negative interference) or were the result of a mutational event conditioned, at least in part, by the difference between the two alleles and perhaps associated with crossing over but not dependent upon it (gene conversion). The ambiguity may be resolved in favor of negative interference if evidence is obtained for the recovery of reciprocal products of the meiotic event, particularly the two alleles in *cis* configuration (double mutant). On the other hand, the ambiguity may be resolved in favor of gene conversion if adequate testing fails to reveal the presence of the double mutant.

In "lower" organisms, particularly the fungi, a tetrad analysis is often possible,

and unequivocal results in favor of gene conversion or negative interference may be available.[5]

However, only the elegant paper of Green[6] has provided unambiguous results in Drosophila. Utilizing the white (*w*) locus in *D. melanogaster*, and in particular the allele $w^{a2}$, Green has demonstrated the recovery of double mutants without recombination of any outside markers, a result most easily interpreted as an example of negative interference, especially since the pseudoallelic nature of the white locus involving the alleles tested by Green is well established. The failure of additional tests to demonstrate that the wild-type flies obtained are accompanied by double mutants leaves the implication that this second class of wild types arises not by multiple crossing over but rather by gene conversion.

Additional data are now available for the garnet locus which indicate beyond any reasonable doubt that at least some of the wild-type flies obtained from testing two different garnet alleles have arisen by a process which does not give reciprocal classes as would be expected from any simple crossing over hypothesis.

*Methods and Materials.*—Five different alleles of the sex-linked gene, garnet, were used. They were *g*, $g^2$, $g^3$, $g^4$, and $g^{53d}$. The first four alleles were obtained independently from different laboratories and are presumably independently arising mutations, but this is not proved. They all result in a brown eye and are phenotypically indistinguishable from each other. The $g^{53d}$ gives an orange-eyed fly which is phenotypically separable from any brown allele. The markers used were wavy wings ($wy^2$), 2.5 units to the left of garnet, and scalloped wings (*sd*), 7.1 units to the right.

Flies were raised in 95 × 25 mm shell vials, utilizing the standard medium made from corn meal, molasses, brewers' yeast, and agar. Methyl parasept, a mold preventative, was added to the medium, and a few particles of powdered live yeast were dusted on the surface of the medium after it had hardened. All cultures were incubated at 25.0 ± 1.0°C.

*The attached-X tests:* The first tests for genetic interaction between the several garnet alleles involved the synthesis of attached-X stocks to provide a partial tetrad analysis. In all attached-X tests one arm of the attached-X chromosome contained the genes $g^{53d}sd$. The other arm contained one of the four brown garnets linked to $wy^2$. In addition, one test was done in which the female was homozygous for $g^{53d}$ and was marked with $wy^2$ on one arm and *sd* on the other. All females so constituted were mated singly by several yellow, scute, apricot, Bar, lozenge ($y^{31d}sc^8$-$w^aB$ $lz^s$) males, hereafter referred to as *FM1*. The attached-X stocks were all synthesized in a similar fashion by utilizing an attached-X $g^{53d}sd/FM1$ triploid stock. To this triploid female was mated a male with an X chromosome marked with $wy^2$ and the desired garnet allele to be tested. Thus, all tests were performed on a similar, if not identical, genetic background.

Whenever a female wild type for garnet was recovered, she was mated singly to several *FM1* males to ascertain her genotype. In addition, several of the sisters of such a wild-type female were mated singly to several *FM1* males to test further the genotype of the mother of the recovered wild-type female. Finally, from the progeny of the wild-type female, a stock was established genetically similar to the original wild-type female for the purpose of obtaining a spontaneous detachment of the attached-X chromosomes.

It was always possible, without great difficulty, to obtain detachments of the attached-X chromosomes. Such detachments were then maintained in stock by mating them as males by yellow (*y*), forked (*f*) attached-X females. It is a simple task to determine that the phenotype of the detached-X chromosome is the same as one of the original X chromosomes of the attached-X wild-type female. The maintenance of such detached chromosomes as males by marked attached-X females minimizes the likelihood of genetic change involving the garnet region.

*The tests of cis to trans:* If garnet is a pseudoallelic locus, it may be supposed that some of the wild-type females have resulted from a reciprocal crossover between the two garnet alleles tested. If this were the case, it would follow that the two garnet alleles, orginally in *trans* configuration, would now be in *cis* configuration. Experimentally, it should be possible to reverse the original procedure and the screen for one and/or the other garnet allele in *trans* configuration.

To test this hypothesis, detached chromosomes presumably containing both garnet alleles and no other markers were made heterozygous with chromosomes containing no garnet alleles and marked with $wy^2$ and *sd*. Since the chromosome which presumably had both garnet alleles resulted in flies with brown eyes, it was necessary only to look for orange-eyed males in the progeny of the heterozygous females just described.

*The retest:* Probably a more conclusive test for the two garnets in *cis* relationship would be to test such a chromosome against another containing only one garnet allele. It would be expected that, if the tested chromosome had both garnet alleles, such a test would fail to yield wild types due to the homozygosity of one or the other garnet allele. However, if it were true that the tested chromosome contained only one garnet allele, and if it were made heterozygous with the other garnet allele, it would be expected that wild types would result in essentially the same manner and frequency as in the original attached-X tests.

To test this possibility, the detached chromosome presumably containing both garnet alleles was made heterozygous with a chromosome marked with $wy^2 g^{53d} sd$. The progeny of such a female, mated to $wy\ g^{53d} sd$ males, were examined for the presence of flies wild type for garnet.

*Genetic test for duplication:* One possible hypothesis to account for the results to be described is the origin, by unequal crossing over, of a duplication for the garnet locus including a wild-type allele. To test genetically for this possibility, detachment of the wild-type complement for attached-X wild-type females was obtained and made heterozygous with a wild-type free-X chromosome. Such heterozygotes might be expected to yield garnet males by similar unequal crossing over, if the original wild-type chromosome were wild-type by reason of containing a duplication.

*Results.—The attached-X tests:* It can be seen in Table 1 that every test involving two different garnet alleles resulted in wild-type females. The frequency of such events approximated one per 20,000 females tested and did not vary significantly from test to test. The only test which failed to yield wild-type females was that in which the garnet allele was the same, namely, $g^{53d}$ in both chromosomes.

TABLE 1

RESULTS OF ATTACHED-X TESTS

(One arm is $g^{53d} sd$; the other arm is $wy^2$ and the garnet allele tested)

| Alleles tested | No. wild-type females recovered | Total no. females tested | Average no./$10^5$ |
|---|---|---|---|
| $g$ | 3 | 58,415 | 5 |
| $g^2$ | 4 | 78,843 | 5 |
| $g^3$ | 5 | 95,139 | 5 |
| $g^4$ | 3 | 50,031 | 6 |
| Total | 15 | 282,428 | 5 |
| $g^{53d}$ | 0 | 539,393 | 0 |

Of greater interest, however, is the genotype of the females recovered. Table 2 contains this information. In general, the females are of two phenotypes: wild-type and wavy. Of the wild-type females the majority (6 of 8) upon progeny test prove to have garnet (*s*) in one chromosome giving flies with brown eyes(and therefore symbolized $g^{br}$) and $wy^2 sd$ in the other chromosome. The wavy females all prove to have $wy^2$ and $g^{br}$ in one chromosome, $wy^2 sd$ in the other.

TABLE 2

PHENOTYPES OF RECOVERED WILD-TYPE FEMALES AND RESULTS OF PROGENY TESTS

| | Genotype of X Chromosomes | | | |
|---|---|---|---|---|
| | Wild-type females | | Wavy females | Scalloped females |
| Alleles tested | $g^{br}/wy^2 sd$ | $wy^2 g^{br}/sd$ | $wy^2 g^{br}/wy^2 sd$ | $sd/g^{53d} sd$ |
| $g$ | 1 | 1 | 1 | 0 |
| $g^2$ | 3 | 0 | 1 | 0 |
| $g^3$ | 1 | 1 | 3 | 0 |
| $g^4$ | 1 | 0 | 1 | 1 |
| Total | 6 | 2 | 6 | 1 |

Such females are most easily interpreted as having arisen due to crossing over between closely linked genes. The results would suggest that $g^{53d}$ is to the left of all of the brown garnets ($g$, $g^2$, $g^3$, $g^4$). This visualization would demand that the wild-type females had resulted from a reciprocal crossover and, moreover, that the chromosome not containing $wy^2$ and *sd* possesses both $g^{53d}$ and the other garnet tested in *cis* configuration. This chromosome, when homozygous, gave females with brown eyes indistinguishable from any of the brown garnets, but it would be folly *a priori* to predict with any certainty the phenotype of a double mutant chromosome.

In addition to the wavy females and the wild-type females described, three other females wild type for garnet were obtained. Two of these females were phenotypically wild type for all of the markers and upon progeny test proved to have $wy^2$ and a brown garnet in one chromosome and only *sd* in the other. To explain such females in a manner similar to that already described requires, in addition to a crossover between the two garnets, a simultaneous crossover between garnet and wavy.

The third exceptional female was homozygous for *sd* and in one chromosome had an orange garnet, most certainly $g^{53d}$. An explanation for this female incorporating a pseudoallelic premise requires a triple crossover: one between wavy and garnet, a second between the two garnets, and a third to the right of *sd*.

*The tests of cis to trans:* If the pseudoallelic notion were correct, then it should be possible to isolate by detachment the chromosome presumptively double mutant, and by proper experimentation to reverse the process and derive from the *cis* configuration of both alleles one or the other, or both, of the alleles depending upon the phenotypic clarity of the test. Since the presumed double mutant chromosome gave brown-eyed flies, only orange-eyed ($g^{53d}$) males were looked for. Table 3 gives the results of this test. No orange-eyed males occurred in over 400,000 male progeny, whereas in the original test (Table 1) fifteen wild types occurred in less than 300,000 flies. The difference between these two tests is statistically significant ($p < 0.01$).

*The retest:* Since it can be argued that the test from *cis* to *trans* is not equivalent to that of *trans* to *cis*, possibly because the critical phenotypes are not as easily distinguishable, or possibly on the theoretical supposition that a "position effect crossing over phenomenon" applies, such that the probability of recombination between the alleles in *cis* configuration is strikingly different from recombination between the same two alleles in *trans* configuration, a more direct test of the presumed double mutant was made. This test involves the assumption that an experiment involving homozygosity of any garnet allele will not yield wild types in appreciable frequency, whereas, if the detached chromosome does, in fact, contain

TABLE 3

RESULTS OF TESTS TO SEPARATE $g^{53d}$ FROM PRESUMED *cis* CONFIGURATION

| Allele test from which chromosome originated | No. of orange-eyed ($g^{53d}$) males recovered | Total no. male progeny |
|---|---|---|
| $g$ | 0 | 68,088 |
| $g^2$ | 0 | 90,104 |
| $g^3$ | 0 | 153,994 |
| $g^4$ | 0 | 115,305 |
| Total | 0 | 427,491 |

only one of the two garnet alleles, the test should yield wild types when such a chromosome is placed opposite a chromosome containing the other allele. Because of the negative results of the *cis* to *trans* test (Table 3), the suspicion was that, if an allele were missing from the double mutant chromosome, it was the $g^{53d}$ allele, and therefore the detached presumably double mutant chromosome was tested opposite a $wy^2g^{53d}sd$ chromosome. If the assumptions are correct, then this experiment, in essence, is a free-X replica of the original attached-X test, and wild types should result in approximately the same frequency. The results in Table 4 bear out this contention. In every test wild-type flies resulted, and although there is a suspicion that the rate (22/684,994) is different from the original attached-X experiments (15/282,428), the difference is not statistically significant ($p > 0.05$).

TABLE 4

RESULTS OF RETESTING PRESUMED *cis* CHROMOSOME OBTAINED FROM ORIGINAL ATTACHED-X TESTS: $g^{bf}/wy^2g^{53d}sd$

| Allele test from which chromosome originated | No. of wild-type flies | Total no. of flies tested | Average no. $/10^5$ |
|---|---|---|---|
| $g$ | 6 | 142,160 | 4 |
| $g^2$ | 6 | 167,646 | 4 |
| $g^3$ | 5 | 229,175 | 2 |
| $g^4$ | 5 | 146,013 | 3 |
| Total | 22 | 684,994 | 3 |

Table 5 lists the phenotypes (and thereby the genotypes) of the wild types recovered. Half of the wild types are *sd*, indicating recombination between $wy^2$ and *sd*, while the other half are $wy^2sd$ indicating that *wy* and *sd* did not recombine, although it is not possible to exclude multiple crossing over, the end result of which is *wy* and *sd* in the original combination.

TABLE 5

PHENOTYPES OF WILD-TYPE FLIES RECOVERED FROM RETESTING OF PRESUMED *cis* CHROMOSOME

| Allele test from which chromosome originated | $wy^2sd$ ♀ | $wy^2sd$ ♂ | $sd$ ♀ | $sd$ ♂ |
|---|---|---|---|---|
| $g$ | 1 | 0 | 5 | 0 |
| $g^2$ | 1 | 1 | 3 | 1 |
| $g^3$ | 1 | 3 | 1 | 0 |
| $g^4$ | 3 | 1 | 1 | 0 |
| Total | 6 | 5 | 10 | 1 |

The data for the alleles $g^2$, $g^3$, $g^4$ in Tables 1, 2, and 3 were previously published.[4]

*Genetic test for duplication:* If the original attached-X wild-type females owed their phenotype to the fact that unequal crossing over had resulted in a duplication for the wild-type locus of garnet (resulting therefore in an attached-X wild type female), it should be possible, by a reversal of the original crossing over phenomenon to recover garnet males from a female heterozygous for the duplication ($+\ g^{53d}\ +$) and a wild-type chromosome. Such heterozygous females were synthesized by obtaining the detachment of the wild-type X chromosome from the original wild-type attached-X females. No garnet males were recovered in 326,240 males tested. Such a negative result suggests that the original wild-type females were not a consequence of unequal crossing over leading to duplication-deficiency phenomena. However, this result by itself does not unequivocally rule out a duplication, nor do

these data exclude the possibility that $g^{53d}$ may itself represent some sort of duplicational phenomenon.

*Discussion.*—As was shown previously,[3, 4] it is possible to obtain flies wild type for garnet when heterozygotes for two presumably different garnet alleles are synthesized. The mechanism causing such wild types remains the principal question raised by the data. The frequent association of recombination with the wild types clearly suggests a causal relationship, and is most simply, and at first approximation, explained by assuming that garnet represents a pseudoallelic locus and that the wild types are the result of crossing over between two closely linked alleles. (Allele is used in this paper to describe noncomplementary genes which occupy either the same locus or very closely linked loci). However, the verification of a pseudoallelic hypothesis, a *cis* arrangement of both garnet alleles, was lacking. Such a result led to the suggstion that garnet might not be a pseudoallelic locus.[4] Since that time the claim has been remade[7] that different garnet alleles can be genetically separated into several closely linked loci, and the evidence to substantiate such an assertion is compelling. The failure of the experiments here reported to prove pseudoallelism does not detract from this claim.

If it is now accepted that the garnet locus is comprised of two or more sites, mutation at any of which will yield a garnet phenotype, why is it that separability could not be obtained in tests with $g^{53d}$, even though wild types for garnet were recovered? The only explanation would seem to be that $g^{53d}$ is a "mutable" allele, to use the original terminology of Demerec.[1, 2] The implication of only $g^{53d}$ as "mutable" is suggested by the data, but the fact of other alleles also "mutating" is not excluded. That mutation in the classical meaning of the word is not the responsible mechanism would be deduced from the facts (*a*) that the rate is too high (1/20,000), (*b*) that nonrandom recombination of outside markers is associated with it, and (*c*) that no wild types were recovered when $g^{53d}$ was homozygous. The term "gene conversion" has been used to describe such recoveries of wild types, and is used in this paper as a descriptive term.

It is also clear that gene conversion of $g^{53d}$ is not simply free, random, nonclassical crossing over within a small chromosome region. The four analyzed wild-type females (Table 2) which appeared at first glance to be probably reciprocal crossovers of two *g* alleles, of which one is $g^{53d}$, proved to be only recombinants between $wy^2$ and *sd*. There was no evidence, from extensive tests, that $g^{53d}$ had moved across to the other *g*-bearing chromosome. Furthermore, while the free-X data in Table 5 show exceptionally high crossing over between $wy^2$ and *sd* in flies which were $g^+$ (11 cases where only 2 would be expected on the basis of the map distance), those crossovers were themselves not randomly distributed in the two regions. All of them were between $wy^2$ and *g*, in spite of the fact that the map shows that crossing over normally occurs three times more frequently between *g* and *sd* than between $wy^2$ and *g*. This strongly suggests some sort of polarity or directional orientation when both gene conversion of $g^{53d}$ and nearby crossing over occur.

The question may be fairly asked whether or not $g^{53d}$ can be separated genetically from any of the other known garnets, i e., whether it is pseudoallelic. Although no evidence is available from these tests to support the proposition of pseudoallelism, it must be re-emphasized that only four females were tested in a definitive way for pseudoallelism. It may be that both gene conversion and recombination between

pseudoalleles are responsible for the occurrence of wild types and that, by chance, the four females tested happened to have arisen by gene conversion. If this were true, then it should be possible to test more wild types arising from attached-X tests and eventually to prove, if the assumptions are valid, pseudoallelism for these garnets.

There are two suggestions, one theoretical, the other experimental, that pseudoallelism is also involved. The experimental result indicates this is the lower frequency of wild-type flies recovered from the free-X test (Table 4) as compared to the frequency in attached-X tests (Table 1). Although the difference is not statistically significant ($p > 0.05$), it is interesting that, in *each* of the four tests of the free-X, the rate of recovery is lower than that in the corresponding attached-X test. Such a result would be expected if wild types are the result, at least in part, of recombination between pseudoalleles. The reason is simply that in free-X tests half of the crossovers between pseudoalleles would result in the double garnet and are probably phenotypically undetectable. In attached-X tests all crossovers result in wild type and thus, if pseudoallelism were the only mechanism responsible, the frequency of wild types recovered in free-X tests would be one half the frequency in attached-X tests. If all the wild types were not due to pseudoallelism, the difference might not be as great as one half.

The theoretical objection to the supposition that pseudoallelism is not involved at all is the difficulty in visualizing any mechanism for gene conversion which does not incorporate some kind of misreplication. (Copy choice would be one such mechanism involving misreplication, but by no means the only one imaginable.) Moreover, this misreplication demands a heterozygosity because wild types are not recovered, or at best are recovered with considerably decreased frequency, when the two alleles tested are identical. Such evidence suggests that a requisite for gene conversion is the availability of a wild-type allele on the homologous chromosome so that a "mistake" may be made. The interesting suggestion in yeast[5] that allelic recombination involves two separate phenomena, DNA replication and chromosome pairing, and thus takes place at two different times, provides a possible answer to allelic behavior at the garnet locus in Drosophila.

*Summary.*—Whenever the mutant $g^{53d}$ is heterozygous with another garnet allele, wild types for garnet are recovered in a low but regular frequency. The critical tests available for proving that such wild types arise through crossing over between pseudoalleles fail in every respect, as does a genetic test for duplication. These failures, plus the unusual nature of a few of the wild types recovered, very strongly suggest that gene conversion is responsible, at least in part, for the occurrence of the wild-type flies.

The author wishes to express his gratitude to Dr. P. T. Ives for reading the manuscript and for his critical suggestions concerning this work. The author is also indebted to Mrs. Phyllis Russell, Mr. Richard Banner, and Mr. Louis Fishman for their technical assistance.

* This work was supported by U.S. Public Health Service research grant C-3114.

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## *SPECIFIC ADENOVIRUS COMPLEMENT-FIXING ANTIGENS IN VIRUS-FREE HAMSTER AND RAT TUMORS**

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*Presented before the Academy, April 22, 1963, by invitation of the Committee on Arrangements*

Although tumors induced in hamsters by adenovirus types 12 and 18 do not contain demonstrable infectious virus, tumorous animals develop high-level complement-fixing (CF) antibodies reactive with homotypic adenovirus tissue culture antigens.[1] Likewise, hamsters carrying transplants of these noninfectious tumors show comparable CF antibody responses.[2] Hamsters which received virus or transplanted tumor tissue, but in which progressive tumor growth did not occur, did not develop CF antibody.[1, 2]

In contrast to human CF antibody responses to adenovirus infections, in which the antibody is primarily formed against the group reactive "A"[3] or "L"[4] soluble antigen, the antibodies in the tumorous hamsters are essentially type-specific and are presumably reactive with antigens similar to the "C"[3] or "E"[4] soluble antigens. Thus, sera from hamsters with tumors induced by type 12 react in high titer with type 12 tissue culture antigen, are somewhat less reactive with type 18 antigen, and give no reaction with the other 26 human adenovirus types. Virus-neutralizing antibodies also develop in hamsters with primary and transplanted tumors, but only in those with highest levels of CF antibody.[1, 2]

This report describes the direct demonstration of virus-specific CF antigens in the noninfectious or "virus-free" hamster tumors induced by adenovirus types 12 and 18, as well as in transplanted tumors[5] and tissue culture cell lines derived therefrom. Also, we describe the induction of tumors in rats by type 12, with comparable findings of noninfectious virus-specific antigens and antibodies in the tumorous animals. Preliminary information on specific CF antigens in hamster tumors induced by SV40 and Rous viruses is also presented.

*Materials and Methods.*—Liter pools of prototype 12 (Huie strain) and 18 (D. C. strain) adenoviruses grown in KB cells, vialed in 1.0 ml quantities, and frozen at −60°C, were used for most experiments in hamsters and rats, and as antigen for neutralization and complement-fixation tests. The titers in primary human embryo kidney (HEK) cultures, observed for 20–30 days or more, were $10^{6.8}$–$10^{7.4}$ $TCID_{50}$ per 0.1 ml for type 12, and $10^{5.4}$–$10^{6.0}$ for type 18. Group complement-fixing antigen titers were 1:16–1:32 for type 12, and 1:8–1:16 for type 18. Viral and antigen pools were also prepared from recently isolated strains of type 12 virus[1] in KB of primary HEK tissue cultures; infectivity titers ranged from $10^{3.8}$–$10^{5.0}$, and group CF antigen titers from 1:8 to 1:16.[6]

*Animals:* Pregnant and weanling Syrian hamsters and Sprague-Dawley rats were obtained from the Animal Production Section of the National Institutes of Health. They were maintained on sterile bedding, in sterile plastic disposable cages, in isolated animal quarters free of polyoma and SV40 viruses.

*Inoculation procedures:* Procedures used for virus inoculation of newborn hamsters have been

described previously;[1] newborn rats were inoculated in a similar manner. Tumor transplants were carried out by inoculating finely minced tumor tissue into suckling or weanling animals, using an 18-gauge trocar. The material was deposited subcutaneously at the nape of the neck. Although tumor transplants readily grew when inoculated by other routes,[1] the subcutaneous site was usually selected because tumors could be observed and measured more readily, and survival time was longer.

*Serum specimens:* All animals were bled at regular intervals from the inner canthus of the eye. Initial bloods were taken 15 days after animals were given transplants and 20–30 days after injection of virus. Subsequent bloods were generally taken at weekly or fortnightly intervals, depending on the progression of tumors. All sera were individually tested for antibody but, for definitive studies of antigen antibody reactions, pools of hamster serums representative of early (lower level) and late (higher level) antibody responses were used.

*Observation of tumor development:* The animals were observed daily, and the onset of grossly observable tumors recorded. Calipers were used to measure the subcutaneous tumors, and the size was recorded as the largest diameter. At autopsy, the tumors were weighed, the dimensions determined, and the gross appearance described; gross and microscopic appearances of the tumors were similar to those reported previously.[1, 2, 7] Aliquots of the specimens were placed in Zenker's fixative for histopathological study; other aliquots were washed in Eagle's basal medium (BME) and used for transplantation experiments, tissue culture preparations, infectivity assay, and/or complement-fixing antigens.

*Tests for viral infectivity:* One-tenth ml of test material was inoculated into 3 tube cultures of primary HEK, maintained in BME with 2% agamma calf serum (Hyland Laboratories, Los Angeles, California). The cultures were observed for 28 days, and the pooled fluids passed to 2 fresh HEK cultures which were observed for 14 days. Tests in our laboratory[8] have shown that prolonged observation of HEK cultures provides the most sensitive isolation system available for adenoviruses. Tests of 51 extracts of primary and transplanted tumors induced in hamsters by type 12 have been uniformly negative, as have tests of 7 rat tumors and of 6 transplanted type 18 tumors. Likewise, repeated tests of nontumorous tissues, urine, and feces of virus-injected hamsters taken at various intervals before and after tumor formation were negative. Additional unsuccessful efforts to demonstrate infectious virus included long-term culture of hamster cells in tissue culture, with tests of the fluids in HEK cultures and growth of trypsinized hamster tumor cells on feeder layers of HEK cells, a method similar to that used for detection of small amounts of SV40 virus.[9, 10]

*Complement fixation tests:* A microtechnique modification of the Bengtson procedure, including overnight fixation at 4°C, was adapted by Sever[11] from Takatsy's microtechnique.[12] In these studies 1.8–2.0 exact units of complement were used for all tests of hamster and rat materials. Three different types of adenovirus preparations were used, as follows:

(1) *"Viral" antigens:* For purposes of this paper "viral" means antigens produced by replicating virus in human or animal tissue cultures. The adenovirus preparations contained soluble antigens as well as viral particles; they were grown in KB cell cultures and consisted generally of supernatant fluids. SV40 antigens were grown in African green monkey kidney cells, and Rous virus antigens were made from tumors produced in the wing web of RIF-free chickens. (2) *"Tumor" antigens:* These represented 10 or 20% extracts of tumor tissues from hamsters and rats. Aliquots of tumor tissue were homogenized in BME at 4°C using mortars and/or Ten Broeck grinders. They were clarified by low speed (2,500 rpm centrifugation for 10–15 min in a refrigerated centrifuge, dispensed in 1 ml amounts, frozen and maintained at −60°C. (3) *"Tumor cell" tissue culture antigens:* Trypsinized hamster tumor cells were serially cultivated in 32-ounce bottles using diploid growth medium with 10% fetal calf serum.[13] After satisfactory heavy growth had been achieved, the cells were scraped from the glass and made into 10% suspensions (v/v) in BME.

*Normal control antigens:* Normal subcutaneous and muscle tissues from hamsters and rats bearing tumors and from normal control hamsters and rats were prepared in the same way as the tumor antigens. Control tissue culture antigens were prepared from uninfected KB and HEK tissue cultures in the same manner as from adenovirus-infected cultures.

All candidate antigens were first checked for anticomplementary (AC) activity. Antigen and antiserum units were based on reactions obtained as follows: "viral" antigen units were determined in chessboard titrations with a human serum pool derived from patients convalescent from

adenovirus infections. This pool, which has been used for a number of years in this laboratory, measures primarily the group-specific adenovirus antigen. "Tumor" antigen units were determined by tests with a pool of serums from hamsters bearing type 12- or type 18-induced tumors.

Antigens in tumor extracts were evaluated (1) by testing them with standard antiserum pools prepared by combining sera from comparable tumorous hamsters, the sera being selected for having high titer CF antibody to the homotypic viral antigen; and (2) by testing serial serum samples from large numbers of individual hamsters against both homotypic and heterotypic viral and tumor antigens. The antigen preparations were routinely used unheated. At least 4 and frequently 8–16 units of antigen (determined by tests with at least 4 units of antiserum) were used for the tests that we describe.

*Results.—Direct demonstration of CF antigens in hamster tumors:* Complement-fixing antigens were readily demonstrated in suspensions of primary and transplant adenovirus-induced tumors when tested with hamster serums exhibiting CF antibody to viral antigens. As can be seen in Table 1, extracts of tumors induced by both adenovirus serotypes gave reactions with a type 12 "positive" hamster serum pool which were equivalent to those obtained with the viral antigens. Serums from hamsters with type 18-induced tumors, and with tumors induced by 3 newly isolated type 12 strains,[1] also reacted with both viral and tumor antigens; although not shown in Table 1, in each case homotypic reactions were higher than heterotypic reactions. No reactions were observed between viral or tumor antigens and normal hamster serums, or between suspensions of normal tissues from either normal or tumorous hamsters and serums positive for viral or tumor antibodies.

Table 2 shows CF antigen titers of a series of extracts of transplanted type 12 tumors in relation to size of tumors and the time interval between injection and harvest. It is striking that all tumors had respectable antigen titers and that all tumors over 10 mm in diameter had antigen titers at dilutions of 1:32 or higher. The majority exceeded 1:32, and of those tested at higher dilutions many had titers of 1:64 and 1:128, levels difficult to achieve in conventional virus growth systems (see *Methods*). The larger tumors in most cases showed anticomplementary (AC) effects at dilutions as high as 1:16. This was associated with necrosis and hemorrhage in the tumors, suggesting that the anticomplementary effects, in part at least, were due to *in vitro* antibody and antigen reactions.

*Antigens in hamster tumor cells cultivated in tissue culture:* A type 12-induced hamster tumor transplant was trypsinized and carried in tissue culture; cell suspensions and extracts, prepared after 3 passages, reacted with the positive type 12

TABLE 1

"CHESSBOARD" COMPLEMENT FIXATION TESTS OF POOLED SERUM FROM HAMSTERS WITH TYPE 12 TUMORS, WITH TYPES 12 AND 18 VIRAL AND TUMOR ANTIGENS

| | | Type 12 serum titer | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 20* | 40 | 80 | 160 | 320 | | 20 | 40 | 80 | 160 | 320 |
| Type 12 viral antigen | 8 | 4† | 4 | 4 | 3 | 1 | Type 12 tumor antigen | 4 | 4 | 4 | 4 | 4 |
| | 16 | 4 | 4 | 4 | 3 | 1 | | 4 | 4 | 4 | 4 | 4 |
| | 32 | 4 | 3 | 3 | 3 | 1 | | 4 | 4 | 4 | 4 | 4 |
| | 64 | 3 | 2 | 2 | 1 | 1 | | 4 | 4 | 4 | 4 | 3 |
| | 128 | 0 | 0 | 0 | 0 | 0 | | 3 | 3 | 3 | 3 | 3 |
| Type 18 viral antigen | 8 | 4 | 4 | 4 | 3 | 3 | Type 18 tumor antigen | 4 | 4 | 4 | 4 | 3 |
| | 16 | 4 | 4 | 3 | 2 | 1 | | 4 | 4 | 4 | 4 | 3 |
| | 32 | 1 | 1 | 1 | 0 | 0 | | 4 | 4 | 4 | 4 | 2 |
| | 64 | 0 | 0 | 0 | 0 | 0 | | 3 | 3 | 3 | 3 | 2 |
| | 128 | 0 | 0 | 0 | 0 | 0 | | 3 | 2 | 1 | 1 | 1 |

* Reciprocal of dilution.
† 4 plus reading on a scale of 0 to 4 = no hemolysis.

TABLE 2

COMPLEMENT-FIXING ANTIGEN TITERS OF 38 TYPE 12 TRANSPLANTED TUMORS* IN RELATION TO SIZE OF TUMOR

| Tumor size and days postinoculation | Number with titers | | | No. pos./no. tested |
|---|---|---|---|---|
| | 1:16 | 1:32 | > 1:32 | |
| <10 mm (12–20 days) | 3 | 2 | 1 | 6/6 |
| 10–19 mm (18–48 days) | 0 | 4 | 9 | 13/13 |
| 20–29 mm (18–31 days) | 0 | 0 | 6 | 6/6 |
| 30–50 mm (26–36 days) | 0 | 1 | 12 | 13/13 |
| Totals | 3 | 7 | 28 | 38/38 (100%) |

* Transplanted to newborns: (antigens = 20% suspensions in BME).

hamster serum pool in titers of 1:16 to 1:32. However, the supernatant fluids from these cultures gave no reaction, suggesting that the antigen is more closely cell-associated than in infected human tissue cultures. A similar culture cell line was supplied to us by Drs. Strohl, Rouse, and Schlesinger, of St. Louis University. This cell line, which originated with a primary hamster tumor induced with type 12, was in 23rd tissue culture passage. Cell extracts also reacted to a titer of 1:16 with the type 12 hamster serum pool. Antiserums from hamsters carrying tumors resulting from injection of cells from the 9th and 12th passages of this tissue culture cell line were also supplied by Strohl *et al.* These serums reacted with types 12 and 18 viral and tumor antigens in a manner indistinguishable from high titered serums of hamsters carrying direct transplants of type 12-induced tumors.[2]

*Cross reactions of the prototype 12 antigen with antiserums from hamsters with tumors induced by recently isolated strains of type 12:* In an earlier communication we reported that 3 recent isolates of type 12 from children induced tumors in hamsters.[1] Despite incubation periods as long as 190 days, hamsters with tumors caused by each isolate developed specific CF antibodies to adenovirus 12 viral antigens. Serum titers versus viral and tumor antigens were equivalent to those produced by the prototype, but reactions with type 18 antigens again were of lesser dimensions. Tumor antigens were also demonstrated directly in transplanted tumors of these recently isolated strains which cross-reacted with serums of hamsters carrying tumors induced by the prototype virus.

*Some properties of the tumor antigens:* The antigen present in tumor extracts did not sediment when centrifuged at 30,000 rpm for 2 hr in the No. 30 Spinco rotor, and was not affected by extraction with ether.

In contrast to the heat stability of the adenovirus group-reactive soluble antigen,[14] the antigens in both tumor and viral preparations which reacted with serums of tumorous hamsters were in part inactivated by heating at 56°C for 30 min (Table 3). The effect of heating was observable primarily with serum taken early after onset of tumor growth, later serums generally showing nearly equal reactivity with heated and unheated antigens. It remains to be determined whether this difference reflects heterogeneity of antigens or differences in reactivity of early and late antibody when tested with partially denatured protein. It should be noted that the late serums also show more cross reactivity between type 12 and type 18 antigens (see below) and are likely to contain virus-neutralizing antibodies.

*Progression of CF antibody responses in hamsters and cross reactions between types 12 and 18:* As noted previously,[2] hamsters carrying tumors induced by adenovirus 12 develop increasingly higher CF antibody titers to viral antigens with the passage of time. We have recorded similar progressions of antibody responses to tumor antigens. In Tables 4 and 5, antibody responses to both antigens of individual hamsters carrying tumors induced by type 12 and type 18 can be compared; each table shows results obtained during 3 successive bleeding periods in a representative experiment with second generation tumor transplants. Those hamsters showing early growth of tumors developed antibodies early, and those showing progressively growing larger tumors in general developed higher antibodies than those with smaller tumors. In most cases, antibodies appeared earlier and

TABLE 3

EFFECT OF HEAT* ON TYPE 12 TUMOR AND VIRAL ANTIGENS

| Antiserums (8 units) | Antigen Titers: Tumor antigens | | Viral antigens | |
|---|---|---|---|---|
| | Unheated | Heated | Unheated | Heated |
| Hamster pool† | 128‡ | 8 | 16 | 4 |
| Human pool† | 0§ | 0 | 32 | 32 |

* 56°C for 30 min.
† See *Methods.*
‡ Reciprocal of dilution.
§ 0 = negative at 1:2 dilution.

TABLE 4

HAMSTER ANTIBODIES* VERSUS TYPES 12 AND 18 VIRAL AND TUMOR ANTIGENS: TYPICAL PROTOCOL (TEST 711), SECOND PASSAGE OF ADENOVIRUS 12 TUMOR TRANSPLANTS†

| Hamster toe clip no. | First Serum—17 Days: Tumor size mm | T-12 V‡ | T-12 T§ | T-18 V‡ | T-18 T§ | Second Serum—26 Days: Tumor size mm | T-12 V | T-12 T | T-18 V | T-18 T | Third Serum—36 Days: Tumor size mm | T-12 V | T-12 T | T-18 V | T-18 T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10 | 20 | 10 | 0 | 0 | 25 | >80 | 20 | 20 | 0 | 45 | >80 | 40 | 40 | 10 |
| 2 | 0 | 0 | 0 | 0 | 0 | 5 | 10 | 0 | 0 | 0 | 10 | 10 | 10 | 0 | 0 |
| 3 | 0 | 10 | 0 | 0 | 0 | 10 | 10 | 40 | 0 | 0 | Died | NT | | NT | |
| 4 | 0 | 0 | 0 | 0 | 0 | 5 | 10 | 10 | 0 | 0 | 15 | 10 | 10 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | <5 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 10 | 0 | 0 | 20 | 40 | >80 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 5 | 10 | 0 | 0 | 0 | 15 | 40 | 80 | 20 | 0 | Died | NT | | NT | |
| 9 | 0 | 0 | 0 | 0 | 0 | — | NT | | NT | | 0 | 0 | 0 | 0 | 0 |
| 10 | 10 | 0 | 0 | 0 | 0 | 25 | 20 | 40 | 10 | 0 | 25 | 20 | >80 | 20 | 0 |

* All sera completely negative when tested versus 8 units adenovirus serotype 2 antigen and normal hamster control antigen.
† Injected in newborn hamsters.
‡ Viral antigens contained 4–8 units.
§ Tumor antigens contained 16 units.

TABLE 5

ANTIBODY RESPONSES OF HAMSTERS BEARING 2D PASSAGE TYPE 18 TUMOR TRANSPLANTS (TYPICAL PROTOCOL—EXP. 791)

| Hamster toe clip no. | First Serum—23 Days: Tumor size mm | T-12 V* | T-12 T† | T-18 V* | T-18 T† | Second Serum—28 Days: Tumor size mm | T-12 V | T-12 T | T-18 V | T-18 T | Third Serum—32 Days: Tumor size mm | T-12 V | T-12 T | T-18 V | T-18 T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 30 | 0 | 0 | 0 | 40 | 30 | 0 | 0 | 20 | 40 | Died | — | — | — | — |
| 2 | 20 | 0 | 0 | 10 | 80 | 30 | 10 | 40 | 20 | >80 | " | — | — | — | — |
| 3 | 25 | 0 | 0 | 0 | 40 | 40 | 10 | 10 | 10 | >80 | 40 | 20 | 10 | 20 | 80 |
| 4 | 25 | 0 | 0 | 10 | 40 | 40 | 10 | 10 | 20 | >80 | 50 | 20 | 20 | 40 | >80 |
| 5 | 25 | 0 | 10 | 10 | >80 | 30 | 0 | 10 | 20 | 40 | 40 | 10 | 40 | 40 | >40 |
| 6 | 15 | 0 | 20 | 0 | 40 | 30 | 0 | 40 | 10 | 80 | Died | — | — | — | — |
| 7 | 15 | 0 | 10 | 0 | 20 | 30 | 0 | 40 | 20 | 80 | 30 | 20 | 80 | 40 | >80 |

* Viral antigen contained 4–8 units.
† Tumor antigen contained 16 units.

reached higher levels versus the homotypic antigens; responses to the heterotypic antigens were less predictable and appeared later and in lower titer. In a few hamsters the heterotypic reactions eventually reached levels equivalent to those obtained for the homotype. Thus, in these hamster tumor experiments, despite the apparently total absence of infectious virus, we have demonstrated expected immunological differences between types 12 and 18 but have also found previously undemonstrated immunological relationships between them. However, considering their various biological similarities, these additional relationships cannot be regarded as wholly surprising.

Analysis of our hamster antibody responses, exemplified by Tables 4 and 5, suggests the possibility of several antigens in the hamster tumors: (1) an antigen reflected in early antibody responses to only tumor antigen which might be similar to a new "cell" antigen, as postulated by Habel and Sjögren for polyoma virus;[17] and (2) another antigen reflected in antibody responses to both viral and tumor antigens, which can better be explained as a direct expression of adenoviral antigen in the tumors. Although not shown in the tables, tissue culture neutralization tests, completed so far only for type 12, have shown similar progressive increases in neutralizing antibodies. Such antibodies in hamsters carrying large virus-free tumor transplants imply replication in the tumors of at least the nucleoprotein responsible for the immunologic specificity of adenoviruses.

*Relation of antibody response to size of tumors:* The data in Tables 4 and 5 suggested a relationship between size of transplanted tumors and the levels of antibodies produced against tumor antigens in hamster serums. Figure 1 confirms this observation for type 12, showing that, although large size was dependent in part on the time interval between inoculation of the transplant and tumor measurement, larger tumor sizes resulted in higher serum titers independent of time. Small tumors which failed to increase in size with time resulted in negative reactions and/or lower titers even in the latest bleeding. Parenthetically, these serial blood specimens were tested with normal hamster tissue antigens (see *Methods*) and with adenovirus type 2 viral antigen; none gave any response at a 1:10 serum dilution. Most of the serums, however, gave reactions with the prototype 12 viral antigens equivalent to those shown with the tumor antigens.

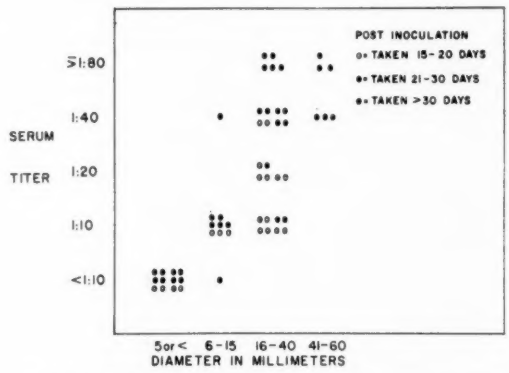


FIG. 1.—Hamster antibody versus adenovirus 12 tumor antigen in relation to size of tumor transplants.

*Tumor induction in rats and demonstration of adenovirus antigens in rat tumors:* Of 10 rats inoculated as newborns with the undiluted prototype adenovirus 12 material, 3 developed tumors. Although the animals received virus both subcutaneously and intraperitoneally, tumors developed only in the peritoneal cavity. The tumors were detected 130–145 days after inoculation, but may have been pres-

ent earlier. The growths were generally massive, appeared to arise from the peritoneal surface, and were histologically identical to the tumors in hamsters.[1,7] Serial transplantation was accomplished in newborn rats but not in weanlings.

Serums of tumor-bearing rats reacted with the types 12 and 18 viral and hamster tumor antigens, and also with the rat tumor extracts (Table 6). Conversely, the rat tumor antigens reacted with the types 12 and 18 hamster serums in titers comparable to the type 12 hamster tumor antigen. Like the hamster material, the rat antigens gave no reaction with the group-reactive human serum antibody.

*Comments on relationships of the specific tumor antigens to the specific viral antigens:* Human adenovirus infections in man generally produce antibodies to the characteristic soluble group-specific antigen regarded as an essential property for the identification and classification of a member of the adenovirus group. Other soluble antigens, usually produced in lesser amounts, can be separated from the group-specific antigens by differential elution on DEAE anionic columns.[3,4] They include the "toxic" or "cell-detaching" "B" antigen, and the type-specific "C" antigen. Studies of purified antigens associated with type 12 have not been reported. However, it is clear that types 12 and 18 strains grown in KB and HEK tissue cultures elaborate a group-specific antigen equivalent to that produced by other adenoviruses[14,15] which is not reproduced by hamster tumors.

The only evidence to date for a "type-specific" antigen in adenoviruses 12 and 18 viral preparations consists of the type-specific reactions reported herein between serums from tumored hamsters and the types 12 and 18 viral antigens. Despite the close similarity in reactivity of the tumor antigens and the specific viral antigen, identity with the "C" antigen cannot be inferred from present data but must await comparative studies with purified antigens from both sources.

Unfortunately, it has not yet been possible to test the tumor antigens against potent type 12 antiserums prepared in nontumorigenic systems. Convalescent serums have been available from only a few known human infections with adenoviruses 12 and 18, and these were from infants who showed very little neutralizing and no CF antibodies to their respective viral antigens.

Evidence favoring a viral genetic determinant of both tumor and viral antigens consists of their similar properties and the progressive development of specific antibodies to both types of antigen by hamsters with adenovirus-induced tumors. The viral origin of the tumor antigens is also suggested by type 12 rat

TABLE 6

ADENOVIRUS ANTIGEN IN RAT TUMORS INDUCED BY ADENOVIRUS 12

| Antigen | | Antigen Titer (Reciprocal) versus Indicated Serum | | | |
|---|---|---|---|---|---|
| Virus | Material | Rat serum, adeno. 12 | Hamster serum, adeno. 12 | Hamster serum, adeno. 18 | Human group-reactive serum |
| Adeno. 12 | Rat tumor 1 | 16 | 16 | 4 | 0* |
| | " 2 | ≥32 | 32 | 4 | 0 |
| | " 3 | 8 | 16 | 4 | 0 |
| | Hamster tumor | ≥64 | ≥64 | 4 | 0 |
| | KB tissue culture | 32 | 64 | 4 | ≥64 |
| Adeno. 18 | Hamster tumor | ≥64 | ≥64 | 64 | 0 |
| | KB tissue culture | 4 | 8 | 4 | 64 |
| Control | Hamster tissue | 0 | 0 | 0 | 0 |
| | KB tissue culture | 0 | 0 | 0 | 0 |

* 0 = less than 4.

tumors which elaborate antigens and stimulate antibodies that have the same properties and react in the same way as those produced in hamsters.

While it is also possible to postulate wholly "new" cellular antigens in the transformed hamster cells which are induced by viral action on cellular genes and not requiring continuous viral information for antigen replication,[16,17] such antigens cannot satisfactorily account for all the immunological phenomena observed in the adenovirus-induced hamster tumors; this is particularly true for the "C"-like CF antibodies and of course the virus-neutralizing antibodies demonstrated in late hamster serums showing broad CF reactions.

*Observations on Other Tumor Cell Antigens.—The papova group tumor viruses:* The first effective use of CF tests in the study of tumor antigens was the work of Kidd and Rous.[18] They found CF, as well as neutralizing, antibody to cottontail papilloma virus in domestic rabbits carrying virus-induced papillomas or transplants of "V" carcinomas derived from papillomas, despite difficulties in demonstrating infectious virus. In later passages of the "V" carcinomas, however, antiviral antibodies disappeared.[19]

Habel and Sjögren *et al.*[17] have reported that mice and hamsters immunized with polyoma virus, another papovavirus, show increased resistance to transplantation of virus-free tumors induced by polyoma virus. They have interpreted this finding as indicating formation of new cellular antigens in the cells altered by the virus. Our efforts to demonstrate new antigens in virus-free polyoma tumors (supplied by Dr. Mearl Stanton, NIH) by means of CF tests with serums of hamsters carrying transplanted polyoma tumors have been positive on occasion, but the reactions were not of the magnitude seen with the other systems described here and cannot be interpreted as specific.

SV40 tumors in hamsters also contain a new cell antigen demonstrable by transplant rejection tests;[20] in addition, much evidence indicates that the tumor cells carry the complete viral genome in a subinfectious state.[21,22]

We are privileged by our colleague, Dr. Paul H. Black, to state that specific complement-fixing antigens can also be demonstrated in SV40 hamster tumors and hamster, mouse, and rabbit cells transformed by SV40 virus,[22] when tested against sera of hamsters with primary or transplanted SV40 tumors. The antigen is continuously present in several clonal sublines of an SV40 transformed hamster cell line and is found in amounts far higher than can be explained by the small amounts of infectious virus demonstrable in each of the clones.

A transplanted SV40 hamster tumor line received from Dr. Melnick showed similar CF reactivity. Early serums from hamsters carrying transplants of this tumor did not react with SV40 tumor antigens, but serums taken several weeks after tumors had reached 15 mm or more in diameter reacted in high titer; however, the hamster serums did not react with tissue culture-grown SV40 viral antigen. Ten tumor antigens reacted in high titer (>1:32) against SV40 positive hamster serum. Although the transplant tumor line is reported to be virus-free,[23] the tumor antigens also showed significant reactions with SV40 convalescent monkey antiserums, also furnished by Melnick, thus suggesting a direct relationship between the SV40-induced hamster tumor antigen and SV40 viral antigens.

*Antigens in Rous sarcoma tumors:* Rubin recently reported the continuous replication of noninfectious Rous virus in chick embryo cells which requires the addition

of Rous-associated virus (RAV) for its expression as infectious Rous virus.[24] Quite recently, we[25] found that tumors induced in hamsters and guinea pigs by the Schmidt-Ruppin strain of Rous virus[26] resulted in the production and continuous transmission on passage of "transplants" of a complement-fixing antigen in the tumors and a corresponding CF antibody in the tumored animals. As in the adenovirus system, those hamsters failing to develop tumors failed to develop antibody. Tumor transplants in hamsters replicated the antigen despite the fact that little or no infectious virus could be found.[27] The antigen was not found in normal hamster tissue, in normal guinea pig tissue, or in chick embryo cells free of RIF virus. However, the antigen was found in various strains of Rous (including the Schmidt-Ruppin strain) and other leukosis viruses grown in chicken tissues and on the chorioallantoic membrane of the developing chick embryo. It was also found in suspensions of a spontaneous subcutaneous "lymphosarcoma" tumor occurring during an outbreak of lymphomatosis in a large flock of chickens.[28]

*Lack of immunological relationships between adenovirus, SV40, and Rous virus hamster tumor antigens:* The three groups of tumor-inducing viruses represented by adenoviruses, SV40 virus, and Rous virus do not share either common or specific viral antigens. Table 7 shows that tumor antigens prepared from hamster tumors originally induced by adenoviruses, SV40, and the Schmidt-Ruppin Rous strain reacted only with serums from hamsters with tumors induced by homologous or closely related viruses.

*Discussion.*—In his concluding address at the 1962 Cold Spring Harbor Symposium on Basic Mechanisms in Animal Virus Biology, Dulbecco[16] commented on evidence concerning "new" antigens in cells transformed by polyoma virus as follows: "At this moment it is impossible to decide whether this new antigen is determined by a gene of the virus persisting in transformed cells, or by a gene of the cell activated by the infection." In the case of adenovirus tumor systems in hamsters and rats, it is not only possible, but necessary, to decide in favor of Dulbecco's first hypothesis, namely, that the CF antigens are formed in response to a viral genome continuously present in the tumor cells.

As noted above, Kidd and Rous 25 years ago used similar serological procedures

TABLE 7

SPECIFICITY OF TUMOR ANTIGEN REACTIONS: CF ANTIGEN TITERS OF TUMOR AND VIRAL ANTIGENS

| Antigen | | Hamster Serums* | | | | |
|---|---|---|---|---|---|---|
| | | Adeno. 12 | Adeno. 18 | SV40 | Ahlstrom | Polyoma |
| Adeno. 12 | Hamster tumor | >64 | 4 | 0† | 0 | 0 |
| " | Rat tumor | 16 | 2 | 0 | 0 | 0 |
| " | "Viral" (KB)‡ | 64 | 4 | 0 | 0 | 0 |
| Adeno. 18 | Hamster tumor | >64 | 64 | 0 | 0 | 0 |
| " | "Viral" (KB)‡ | 8 | 4 | 0 | 0 | 0 |
| SV40 | Hamster tumor | 0 | 0 | 16 | 0 | 0 |
| " | "Viral" (AGMK)§ | 0 | 0 | 0 | —† | —† |
| Ahlstrom | Hamster tumor | 0 | 0 | 0 | 32 | 0 |
| Polyoma | Hamster tumor | 0 | 0 | 0 | 0 | 0** |
| Control | Hamster tissue | 0 | 0 | 0 | 0 | 0 |
| " | KB | 0 | 0 | 0 | 0 | 0 |

* Serum of hamsters bearing transplanted hamster tumors, used at 1:20.
† 0 = antigen titer less than 4; — = not tested.
‡ Grown in KB tissue culture.
§ Grown in African green monkey tissue culture.
** Tests of other polyoma tumor antigens have given suggestive positive reactions with serum from hamsters with transplanted polyoma tumors.

to demonstrate papilloma virus persisting in virus-induced domestic rabbit papillomas and the carcinomas that usually emerged. The fact that the tumored rabbits developed neutralizing and CF antibodies to the cottontail papilloma virus was cited as evidence for viral activity in a "masked" state.[18,29] This concept was supported recently when Ito demonstrated infectious papilloma DNA in the domestic rabbit papilloma.[40] Although the data presented in this paper on specific CF antigens in adenovirus-induced tumors imply the presence of inheritable genetic information from the virus, it does not permit us to decide whether the antigens themselves are "inducers" or merely "indicators" of the viral oncogenic process.

*Implications for etiology of human cancer:* The similar but immunologically distinct antigens found in "virus-free" hamster tumors induced by adenoviruses 12 and 18, SV40, and avian sarcoma viruses suggest that specific CF antigens of various viral origins might well be looked for in the generality of naturally occurring animal and human cancers, despite numerous failures to detect infectious viruses.

*Summary.*—Type-specific complement-fixing antigens were demonstrated in cancers induced in hamsters by adenovirus types 12 and 18, and in rats by adenovirus type 12. Primary and transplanted tumors were found to be devoid of infectious virus in over 50 separate tests. The specific CF tumor antigens occurred with remarkable regularity in transplanted types 12 and 18 tumors and in serial tissue cultures of type 12 hamster tumor cells. The tumor antigens were not sedimentable and were found to have other properties similar to type-specific or "C-soluble" antigens produced in tissue cultures during replication of adenoviruses 12 and 18. Antibodies induced in hamsters and rats carrying primary tumors and serial transplants of tumor cells reacted with type-specific viral antigens produced in KB and HEK tissue cultures by replicating adenovirus types 12 and 18.

The continuous presence of type-specific viral-like antigens in the absence of the virus itself provided high-order evidence in favor of the conclusion that they were coded for by viral information incorporated in the genetic apparatus of the cancer cells. Alternative hypotheses postulating an inheritable "new" cell antigen induced by virus action on cellular genetic material was not excluded, but such an antigen could not explain all the phenomena observed, particularly the virus-neutralizing antibody produced in hamsters carrying large adenovirus-induced tumors for long periods. Complement-fixing antigens were also found in hamster tumors produced by the SV40 virus, and the Schmidt-Ruppin variant of avian Rous virus. These virus-like tumor antigens, like the adenovirus antigens, could not be accounted for by demonstrable infectious virus.

The presence of specific virus-related antigens in hamster and other animal tumors induced by 3 different groups of viruses (adenovirus, papovavirus, and avian leucosis) suggested that similar antigens might be demonstrable in some naturally occurring "virus-free" tumors of animals and man.

* Presented during the Symposium on Virus-Cancer Relationships, Wendell M. Stanley, *Chairman*, under the title "Evidence for the Intrusion of Adenovirus 12 Genetic Information in the Genome of Induced Tumor Cells."

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# *ANEUPLOIDY IN THE DEGENERATIVE PHASE OF SERIAL CULTIVATION OF HUMAN CELL STRAINS**

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*Communicated by Warren H. Lewis, June 26, 1963*

The factors affecting chromosomal stability of mammalian cells in long-term culture are not well understood, aside from the effects of experimentally applied agents such as irradiation and viruses. Species differences[1–3] and perhaps cell types appear to be important with respect to the occurrence of spontaneous alteration or heteroploid transformation.[4, 5] Cells cultured from mouse tissues seem to be extremely labile with regard to chromosome changes and associated morphologic and growth alterations.[1, 6, 7] On the other hand, in metaphase studies to date, cultured human cells of the fibroblastic type remain diploid and show no tendency to undergo spontaneous transformation.[8–11]

Interest in spontaneous occurrences of *in vitro* transformation of mammalian cells has been based upon hope for its confirmation as a model of the *in vivo* process of malignant change. The two processes have many features in common,[5, 12] and early proposals that viruses present in the original tissue or serum might be causative agents have been strengthened by the numerous studies showing that certain viruses do produce transformation *in vitro*. Experiments with SV40 virus and human cells have provided another reproducible cell-virus system for the study of transformation throughout its course.[13, 14] Furthermore, the discovery of chromosome lesions in human cells in association with measles infection[15] has increased our interest in the nature and extent of chromosomal changes which occur *in vitro* in the absence of any known agent.

This report is concerned with a study of metaphase chromosomes in two human cell strains during their total period of *in vitro* cultivation. In a previous less extensive study no aberrations were seen in various human diploid cell strains in material from the 9th to the 40th subcultivation *in vitro*.[10] In the present study spontaneous chromosome changes were encountered, but only in those cultures which had been subcultivated for 40 or more times, i.e., during the degenerative phase of their characteristically limited *in vitro* life. Although this aneuploidy seems to be associated with the degenerative period in long-term cultivation, on no occasion did transformation in terms of altered morphology, growth rate, or capacity for indefinite cultivation occur among many parallel cultures of these two strains. In long-term cultures of human fibroblasts Sax and Passano [16] have previously shown an association between "age *in vitro*" and increase in spontaneous rate of anaphase aberrations.

*Materials and Methods.—Cell strains:* Cell strains WI-26 and WI-38, male and female, respectively, were derived from fetal lung tissue by L. Hayflick according to procedures previously described.[10] In essence, cultures were grown as monolayers of fibroblastic-like cells in Eagle's basal medium with 10% calf serum and 50 μg aureomycin per ml and subcultivated by trypsinization with 0.25% trypsin (Difco 250:1). Subcultivation from one to two milk-dilution bottles was performed twice weekly, i.e., when the monolayer had become confluent. Confluency was achieved every 3–4 days during the major period (4–5 months) of total *in vitro* cultivation. After approximately 35 total passages *in vitro*, cultures required longer periods (5–8 days) to achieve confluency;

eventually (50 ± 10 passages), all cultures of these diploid cell strains failed to replicate sufficiently to permit any further subcultivation. It is recognized that each subcultivation or "passage generation" is only an approximation to cell population doubling, and the total number of passages has no significance except as a crude measure of the *in vitro* stage any strain has reached. The terminal portion of the finite period of serial subcultivation is referred to as Phase III[10] or as the degenerative phase, and in the absence of heteroploid transformation this limitation on cultivation has long been recognized. Ampoules containing $2 \times 10^6$ cells were frozen and stored in liquid nitrogen by procedures described previously.[10] Substrain designations in Tables 1 and 2 refer to cultures reconstituted from such ampoules and carried independently.

*Chromosome preparations:* Chromosome studies were made from permanent mounts of Giemsa stained air-dried metaphase preparations.[17] Ordinarily cells were harvested with trypsin on the second day after subcultivation, following 3–5 hr of treatment with Colcemid (CIBA), 0.05 μg per ml of medium. The cell suspension was concentrated in ½ ml of trypsin solution, and a 4-fold volume of distilled water was added to swell the metaphase cells hypotonically for 8–10 min. Fixation was made with 3:1 methanol:acetic acid. Spreading was done by ignition of a drop of fixative, containing cells in suspension, immediately after its application to the surface of a clean wet slide. Suitable metaphases were selected under low power (150×) observation, and those judged to be free from excessive spreading were then studied under oil immersion optics. In all cells so selected, the chromosomes were counted, and 25–40% of the metaphases of each sample were subjected to detailed karyotypic analysis, involving the identification of individual chromosome pairs or groups of the human karyotype according to the Denver convention: Nos. 1, 2, 3, 4–5, 6–X-12, 13–15, 16, 17–18, 19–20, 21–22-Y. Metaphase counts considered to be artifacts because of scattering of some chromosomes or because of accidental contamination of one metaphase with chromosomes of another were not excluded from the data on the various samples studied. For each determination of the level of tetraploidy existent in the dividing cell population 250–300 unselected metaphases were examined and roughly estimated as being diploid or tetraploid.

*Observations: WI-26:* Chromosome counts, numbers of cells analyzed, and other karyologic observations from seven different cultures of strain WI-26 are presented in Table 1.

Chromosome preparations from diploid cell strain WI-26 were first made at its 19th subcultivation passage. In a sample of 100 cells the exact chromsome number was determined, and 36 cells were analyzed in detail. It could be seen that most of the cells had a normal male chromosome complement, and in the hypodiploid cells no pattern concerning the missing chromosomes could be deduced. Of the dividing cell population 3.1% were tetraploid, and no abnormal chromosomes were found.

Between the 28th and 37th *in vitro* passages, 106 cells were examined, and of these 28 were analyzed in detail. A normal distribution of chromosome numbers, skewed toward hypodiploid counts, was observed. The analysis of each of the 9 hypodiploid cells of the sample did not reveal any consistency as to the chromosomes which were missing, and these counts are presumed to be artifacts. A somewhat higher value of tetraploidy was recorded at the 28th passage, and again at the 37th passage, 4.5 and 4.4%, respectively. In the sample from the 32nd passage, 1.6% of the dividing cells were tetraploid. An acentric fragment was observed in one of 45 cells examined at the 37th passage. It was found in a cell with 46 (excluding the fragment) chromosomes, which was lacking a No. 4–5 chromosome and contained an extra chromosome in the 6–12 group. It is possible that the fragment resulted from breakage in the long arm of a 4–5 chromosome; this deficient chromosome would then be indistinguishable from members of the 6–12 group. Thus, from a total of over 200 cells counted in cultures of the 19th, 28th, 32nd, and 37th passages, of which 64 were studied in detail, only one cell was found which had an abnormality.

Between the 41st and 54th *in vitro* passage, 58 cells of WI-26 were karyologically examined. Six of 25 metaphases at the 41st passage contained abnormal dicentric chromosomes, while 3 out of 20 did so at the 54th passage. A small sample of 13 cells from 43rd passage material revealed no abnormalities, and the extremely low frequency of mitosis prevented extension of this sample. At the 41st passage, 3.6% of the cells were tetraploid, and the very high value of 16% tetraploidy occurred in the 54th passage material.

Substrains of WI-26 (X, XI, XIII, XIX, and XXIII) could not be subcultivated more than 40–49 total passages; XXVI survived for 56 passages *in vitro*.

## TABLE 1

### Chromosome Count Distribution and Karyologic Data from Human Diploid Cell Strain WI-26

| *In vitro* passages | WI-26 substrain designation | Chromosome Counts: < | 43 | 44 | 45 | 46 | 47 | 49 | 72 | 76 | 91 | 92 | Tetraploidy % | Identity of "Missing" Chromosomes: 1–5 | 6-x-12 | 13–15 | 16 | 17–18 | 19–20 | 21–22-Y | Total cells | Analyzed cells | Types of Abnormal Chromosomes: D | f | m | other |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19th | X | 1 | 1 | 2 | 5 | 88 | | | 1 | | | 2 | 3.1 | 1 | 2 | 1 | | 1 | | 2 | 100 | 36 | | | | |
| 28th | XI | 1 | 1 | | 2 | 16 | | | | | | | 4.5 | 1 | 2 | | 1 | 1 | | 1 | 20 | 4 | | | | |
| 32nd | XIX | | | 1 | 1 | 38 | 1 | | | | | | 1.6 | 1 | 2 | | | | | | 41 | 12 | | | | |
| 37th | XXIII | | | | 3 | 42 | | | | | | | 4.4 | 1 | | 1 | 1 | | | | 45 | 12 | | 1 | | |
| 41st | XXIII | 1 | | 1 | 4 | 19 | | | | | | | 3.6 | 1 | 4 | 2 | | | 2 | | 25 | 12 | 6 | | | |
| 43rd | XIII | | 1 | 1 | 1 | 10 | | | | | | | | | | | | | | 1 | 13 | 5 | | | | |
| 54th | XXVI | | | 1 | 5* | 12† | | | 1 | 1 | 1 | | 16.0 | | 2 | | | 2 | 1 | | 21 | 6 | 4 | | | |

* Cell presumed 13/21 type translocation.
† Pseudodiploid cell; monosomic and trisomic.
D = Dicentric; f = acentric fragment; m = minute.

## TABLE 2

### Chromosome Count Distribution and Karyologic Data from Human Diploid Cell Strain WI-38

| *In vitro* passages | WI-38 substrain designation | Chromosome Counts: < | 43 | 44 | 45 | 46 | 47 | 53 | 64 | 92 | Tetraploidy % | Identity of "Missing" Chromosomes: 1–5 | 6-x-12 | 13–15 | 16 | 17–18 | 19–20 | 21–22 | Total cells | Analyzed cells | Types of Abnormal Chromosomes: D | f | m | other |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4th | P | 2 | 1 | 3 | 7 | 183 | 3‡ | | 1 | | 1.0 | | 8 | 1 | 1 | 3 | 4 | 3 | 200 | 84 | | | | |
| 14th | II | | | | | 19 | | | | 1 | 0.8 | | | | | | | | 20 | 9 | | | | |
| 21st | P | | | | 1 | 21 | | | | | | | 1 | | | | | | 22 | 10 | | | | |
| 31st | IV | | | 1 | 2 | 47 | | | | | 1.0 | 1 | | 1 | | | 1 | | 50 | 22 | | | | |
| 33rd | V | | | 2 | 3 | 46† | | | | | 0.4 | 2 | 2 | 1 | | | | 1 | 51 | 19 | | | | |
| 35th | V | | | | 2 | 28 | | | | | | | | | | | | | 30 | 10 | | | | |
| 37th | IV | | | 1 | 5 | 43 | | | | 1 | 3.8 | 1 | 2 | 2 | | | | 1 | 50 | 18 | | | | |
| 41st | P | 1 | 2 | 4 | 9* | 87† | 1 | | | | 2.7 | 8 | 4 | 5 | 2 | 4 | 3 | 3 | 104 | 51 | 3 | 1 | | |
| 41st | IV | | 1 | 1 | 6* | 30† | | 1 | | 1 | 5.1 | 5 | 2 | 5 | 1 | 1 | | 4 | 40 | 18 | 2 | 1 | 1 | 2 |
| 46th | II | 1 | 1 | | 7 | 11 | | | | | | 4 | 2 | 3 | 2 | 1 | 1 | 3 | 21 | 10 | 6 | | | |

* Cell with presumed 13/21 type translocation.
† Pseudodiploid cell; monosomic and trisomic.
‡ Artifact due to contaminating chromosome from another cell, two cases.
P = parental culture; D = dicentric; f = acentric fragment; m = minute.

*WI-38:* At the fourth subcultivation passage 200 WI-38 cells were examined and, of these, 84 were subjected to detailed karyotypic analysis. The distribution of the exact chromosome counts obtained is given in Table 2. Among 14 hypodiploid cells no consistent pattern as to the identity of the missing chromosomes could be determined. Three cells with 47 chromosomes were observed, but in two of these contamination from another metaphase was definitely indicated by a differential state of condensation in the extra chromosome. In all other cells examined, no deviation from the normal female karyotype was observed. At this early stage *in vitro*, the frequency of tetraploid cells in the dividing population was 1.0%.

Essentially similar findings were obtained concerning the chromosomal constitution of various WI-38 substrains from the 14th to the 37th passage; 223 cells were examined and 98 of these karyotypically analyzed. A single pseudodiploid cell was observed in the 33rd passage sample. The level of tetraploidy remained low, except for one slightly higher value at the 37th passage, 3.8%.

From WI-38 cultures between the 41st and 46th *in vitro* passages, the chromosomes of 165 cells were counted, and 79 of these were karyotypically analyzed. Aneuploid changes and a marked increase in frequency of hypodiploid cells are evident in all of these late passage samples. Although no obvious pattern as to the missing chromosomes can be observed (Table 2), the fact that 2% (41st passage) to 30% (46th passage) of the cells exhibited obviously abnormal chromosomes would imply that perhaps many of the hypodiploids were not artifacts of technique. The abnormalities observed were mainly dicentrics formed by translocations between two chromosomes of the complement, in some cases identifiable chromosomes. Less frequently (Table 2), fragments, minute chromosomes, and abnormal monocentric chromosomes were observed. In this late passage WI-38 material, the frequency of tetraploid cells (2.7–5.1%) was elevated above values seen in lower passage levels (Table 2). The original or parent strain of WI-38 was cultivated for a total of 48 passages, and none of the three substrains (II, IV, V) could be subcultivated more than 43 to 48 passages *in vitro*.

*Discussion.*—Aneuploid changes appeared in different substrains of each strain studied following a long period of apparent chromosome stability. The earliest chromosome aberrations were coincident, with the beginning of the decline of each strain during its serial subcultivation. Setting aside the question of criteria for chromosomal or karyotypic "normality," we must consider whether these observations are generally applicable to human fibroblast strains in long-term culture. Since strains WI-26 and WI-38 were selected for extended investigation for the sole reason that one is male and the other female, it seems warranted to regard them as representative of such serially propagated strains, at least for those of embryonic origin. Further, two other human fibroblast strains cursorily examined also showed some aneuploid cells at high passage levels of cultivation.

That our previous study[10] failed to reveal aneuploid changes with increased "culture age" is attributed to the fact that only two of the 16 samples studied from 13 strains were from cultures approaching or in the period of decline, i.e., above the level of the 35th passage. At its 40th passage, WI-12 revealed no abnormalities in a sample of 33 metaphases, and only 17 cells were available from the 39th passage of WI-1 because of the low growth rate in its declining phase.

No aneuploidy was observed in earlier studies on long-term cultivated human fibroblasts. Among cultures from individuals 1–41 years of age, Tjio and Puck[8] reported "no variation in chromosome number and morphology." Their cultures were studied over periods comparable to those reported in the present paper. In a later paper[9] these authors remarked upon the constancy of the human karyotype, finding no change in number or morphology over "20 successive harvests (transfers). . . involving more than 40 generations." Makino and co-workers[11] reported the maintenance of a "normal complement of 46 chromosomes" in cells obtained

from the 2nd to the 44th subculture; however, they also state, "In comparison with the results of chromosome counts in the primary cultures, cells with hypo- or hyper-diploid chromosome numbers occurred at a higher incidence in the subcultured specimens."

These authors did not present details concerning the proportion of their large total sample which was derived from the late-passage cultures mentioned. On the other hand, in this study we deliberately sought information during the period when the cultures' growth rates had begun to diminish. This fact may account for our observing aneuploidy, whereas they reported none. Our results, in respect to samples taken prior to about the 40th subculture, are in accord with the other studies. The constancy of the human karyotype in such material is impressive when compared to continuously cultivatable cell lines from mammalian tissue which are near-diploid, i.e., containing a large number of apparently euploid cells but with some pseudo-, hypo-, and hyper-diploid cells also detectable.[2]

For *a priori* reasons, criteria for the "normal" human karyotype should be based upon *in vivo* dividing populations (such as direct preparations from bone marrow) or upon primary or very early tissue cultures. This must be determined against a background of a certain amount of hypodiploid counts which, for technical reasons, are spurious. Within these limitations of technique and of the experience of the investigators, prior to the 40th passage level, both strains WI-26 and WI-38 may be regarded as normal or classic diploid. A minor reservation may be made with respect to strain WI-26, however, as the tetraploid percentages observed at the 28th and 37th passages were slightly above values usually observed. A number of studies[8, 10, 11] have shown that the tetraploidy level in presumably normal cultures of human fibroblast cells is seldom greater than 3 per cent. An increase in proportion of tetraploids in the metaphase population is a common feature of SV40 transformation of human fibroblasts.[18, 19] We have observed a similar association between tetraploidy increase and subsequent spontaneous heteroploid transformation in serially cultivated cells of the rhesus monkey.

Considerations as to the tissue's species of origin and the possible presence of inapparent viruses may explain the fact that adult rhesus monkey kidney tissue cultures ordinarily degenerate within a few weeks, surviving no more than 3 or 4 subcultivations. These monkey kidney subcultures may show an extremely high frequency of mitotic aberrations (16–40 per cent) even before the second week of *in vitro* cultivation.[20] That human cell types other than fibroblasts may show entirely different patterns with respect to their fate *in vitro* is well known. For example, extra-embryonic amnion can usually be cultivated for only a few subdivisions before it degenerates or, on occasion, transforms. It is possible that the inherently limited *in vivo* growth potential of amnion, a kind of "tissue age," may be expressed even *in vitro*.

The three WI-26 substrains (XIII, XXIII, XXVI) which revealed aneuploidy survived as serial cultures no longer than various parallel subcultures from this strain which were not examined for chromosome changes. The same applies to the particular substrains (Parental, II and IV) comprising the post-40th passage material in which aneuploid changes were observed in the WI-38 strain. Among the rearrangements and chromosome breaks observed, there was no consistency as to the particular chromosomes affected.

We cannot exclude the possibility that the two particular strains, WI-26 and

WI-38, were in some way predisposed to undergo aneuploid changes or that some unknown effect of the medium used has induced an instability. However, aneuploid changes were noted in different substrains, even though they were carried on medium from independent commercial sources. It therefore appears more likely that our findings may apply generally to such cells and confirm the study by Sax and Passano[16] in which anaphase bridges, lagging chromosomes, rod and dot deletions were found to increase approximately 3-fold during six months of serial cultivation of human fibroblast strains. Interphase nuclei with abnormal sizes and shapes are seen in daughter cells in later passage material[10] and are presumably the products of abnormal division.

The conditions in these human fibroblast cultures, after loss of their proliferative capacity, are superficially identical to those reported in cultures of mouse cells just prior to the usual reversal of the latter's declining growth rate.[1, 7] This increase in growth rate then leads to establishment of the mouse cell culture as a continuously propagated and usually heteroploid cell line. In spite of the degenerate state of late passage cultures of strains WI-26 and WI-38, with chromosome aberrations and reduced mitotic activity, none has undergone a spontaneous transformation, although numerous cultures have been observed 1–3 months after cessation of growth.

*Note added in proof:* It has come to our attention that M. C. Yoshida and S. Makino have reported quite similar findings in the *Japan. J. Human Genetics*, **5**, 39 (1963). These independent observations strengthen our conclusion that the presence of aberrations associated with *in vitro* decline of such cell strains is a general phenomenon.

* Supported in part by USPHS research grant CA-04534, contract PH-43-62-157, and career development award 5K3-CA-18,372 from the National Cancer Institute. The excellent technical assistance of Miss P. Mancinelli and Mrs. M. Lebowitz is gratefully acknowledged.

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# *COMPUTER-COMPILED OCEANOGRAPHIC ATLAS: AN EXPERIMENT IN MAN-MACHINE INTERACTION**

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*Communicated June 24, 1963*

We are exploring concise modes of representation of the total accumulated oceanographic station data which is becoming available in standardized punched-card format at the National Oceanographic Data Center.

Conventional representation of oceanographic data is in the form of atlases of horizontal charts, vertical profiles, characteristic diagrams, etc., of such properties as temperature, salinity, specific volume anomaly, geopotential anomaly, dissolved oxygen concentration, etc. The most celebrated examples are the handsomely produced colored plates of the German *Meteor* expedition (1925–1927). However, this method, appropriate to 310 oceanographic stations, needs to be supplemented by methods capable of rapidly handling the entire body of stations on record—of the order of 300,000; witness the fact that at present, five years after the IGY cruises, the oceanographic data collected then has been only partially analyzed. Furthermore, in preparing a printed atlas certain irrevocable choices of scale, of map projection, of contour interval, and of type of map (shall we plot temperature at standard depths, or on density surfaces, etc.?) must be made from the vast infinitude of all possible mappings. An atlas-like representation, generated by digital computer and displayed upon a cathode-ray screen, enables the oceanographer to modify these choices at will. Only a high-speed computer has the capacity and speed to follow the quickly shifting demands and questions of a human mind exploring a large field of numbers. The ideal computer-compiled oceanographic atlas will be immediately responsive to any demand of the user, and will provide the precise detailed information requested without any extraneous information. The user will be able to interrogate the display to evoke further information; it will help him track down errors and will offer alternative forms of presentation. Thus, the display on the screen is not a static one; instead, it embodies animation as varying presentations are scanned. In a very real sense, the user "converses" with the machine about the stored data. In the belief that the only practical way to illustrate this ideal of man-machine relationship is to produce a working—if still primitive—model, we have completed a pilot display program using a PDP-1 computer with a magnetic tape unit, a cathode ray tube visual display unit, a light pen, an input-output typewriter, and control switches. Oceanographic data is transferred from the N.O.D.C. cards to magnetic tape programs. In operation, a map appears on the screen, the specific geographical area (e.g., in Figure 1, the South Atlantic) having been designated by typed instructions. The operator may then select a subarea within the area for enlargement and display in the same manner, and may continue this process until the desired degree of enlargement has been obtained.

By typing an appropriate symbol, the computer transfers the data for the selected region from magnetic tape storage into the computer memory, and various forms of display then appear.

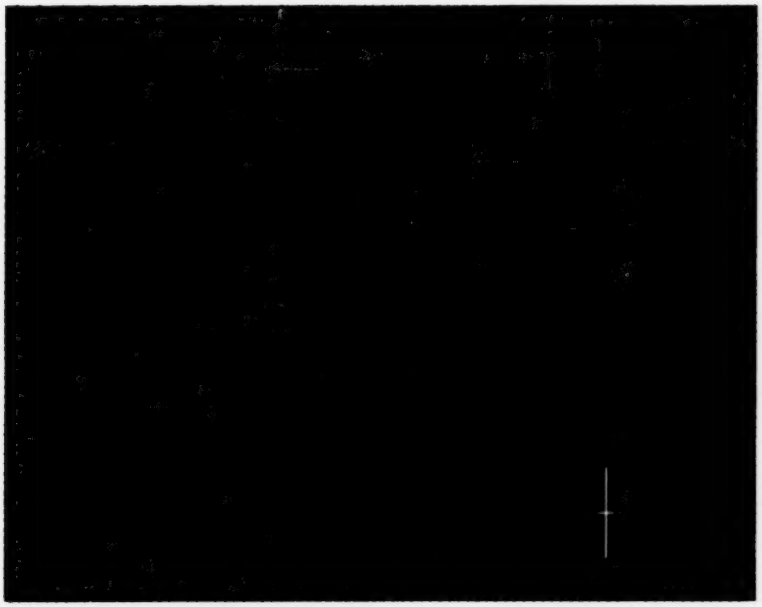

FIG. 1.—A display of *Meteor* stations, showing the position of the 15°C temperature contour at 200 meters by the two kinds of dots: diamonds are stations with temperature equal to or greater than 15.00°C; small individual dots are stations with temperature less than 15.00°C. In use the temperature is varied at will.

Thus, in Figure 1, we are looking at a chart of temperature at 200 meters depth in the South Atlantic Ocean as given by the *Meteor* data. The temperature scale has been set at 15°C. Stations reporting temperatures of 15° or *higher* at a depth of 200 meters appear as diamonds on the visual display scope. Stations reporting temperatures *lower* than 15° appear as small single dots. The temperature scale can be moved as rapidly as the user desires by pushing the illuminated crosspiece (below the temperature) up or down with the light pen. In this way, a varying "temperature contour" is outlined, as the boundary between diamonds and small dots changes to correspond to the momentary value of indicated temperature.

The light pen can be pointed at a station on the scope to make the station number appear. Charts of other variables at other depths and in other areas can be called up on the display at will. In addition, series of photographs such as this can be used as a source from which to draw contour maps for a more permanent form of publication.

In Figure 2 the scale has been enlarged so that we are looking at the central region of Figure 1. The depth is changed to 2,000 meters. The temperature happened to be 3.32°C at the moment the photograph was taken. In addition to *Meteor* stations, IGY stations of the *Crawford* and *Atlantis* are also displayed. They are distinguished from *Meteor* stations by their very regular linear arrangement along parallels of latitude, and also by replacing diamonds by squares where the temperatures are 3.32°C or greater. One can immediately see how closely the older *Meteor*

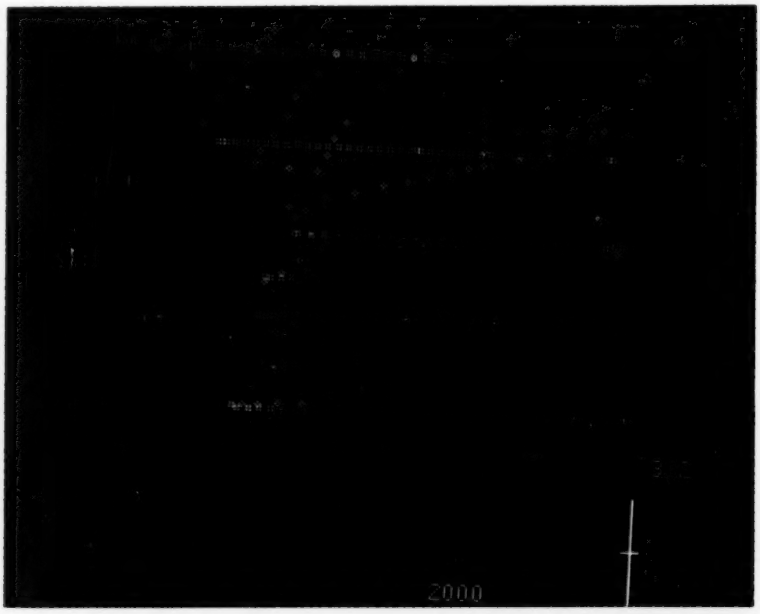


FIG. 2.—IGY data superposed upon *Meteor* data in smaller area. Temperature is set at 3.32°C; depth at 2,000 meters. See text for explanation.

temperatures and the newer IGY temperatures agree, but of course the most convincing demonstration is to watch the display as the temperature is varied.

The dot display enables the user to perform mentally the equivalent of contouring without abandoning the original data. It is the most practicable way we know of presenting original data in an easily comprehended summary-pattern.

The present display program includes not only temperature, but also salinity, oxygen, specific volume anomaly, and dynamic height as well, and it will produce similar displays for any of the 300,000 stations on record at N.O.D.C. We expect to extend the types of display to include charts of properties on isentropic surfaces, core-layers, vertical profiles, individual station soundings, temperature-salinity curves, and other characteristic diagrams, and to make the program available at the computation laboratory at M.I.T. to students and research workers as a tool for exploring the ocean by themselves.

* Supported by NSF grant G-19696.

# *THE INTEGRATION OF THE FIRST-ORDER PERTURBED WAVE EQUATION FOR EXCITED STATES OF ONE-DIMENSIONAL SYSTEMS**

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*Communicated May 27, 1963*

Recently there has been a renewed interest in perturbation theory in quantum mechanics. One aspect of this development, due largely to Dalgarno and his colleagues,[1] has been the successful calculation of the first-order corrections to the expectation values of one-electron operators. This has been achieved by obtaining the exact solutions of the first-order wave equations rather than by attempting to use the very awkward representation of the first-order wave function in terms of the spectrum of solutions of the unperturbed Hamiltonian.

It was first pointed out by Young and March[2] that the first-order wave equation for a one dimensional system can always be integrated by quadrature. However, these authors only discussed the ground state of the system in which the wave function has no nodes. In the case of excited states, singularities occur in the equation owing to the nodes of the unperturbed wave function. The object of this paper is to show how the singularities may be handled both analytically and in numerical work.

*First-Order Wave Equations.*—For a system with the Hamiltonian $H = H_0 + V$ the perturbation expansions of the exact wave function $\psi_n$ and the exact energy $E_n$ of the nth state may be written

$$\psi_n = \psi_n^{(0)} + \psi_n^{(1)} + \ldots, \tag{1}$$

$$E_n = E_n^{(0)} + E_n^{(1)} + E_n^{(2)} + E_n^{(3)} + \ldots, \tag{2}$$

where $\psi_n^{(0)}$ and $E_n^{(0)}$ are the nth normalized eigenfunction and eigenvalue (assumed nondegenerate) of the unperturbed Hamiltonian $H_0$ given by $(H_0 - E_n^{(0)})\psi_n^{(0)} = 0$. The equation for the first-order wave function $\psi_n^{(1)}$ is

$$(H_0 - E_n^{(0)})\psi_n^{(1)} + (V - E_n^{(1)})\psi_n^{(0)} = 0, \tag{3}$$

and therefore the first-order energy is given by $E_n^{(1)} = \langle\psi_n^{(0)} V \psi_n^{(0)}\rangle = V_n$. The second-and third-order energies are given in terms of $\psi_n^{(1)}$ by

$$E_n^{(2)} = \langle\psi_n^{(1)}(V - V_n)\psi_n^{(0)}\rangle, \tag{4}$$

$$E_n^{(3)} = \langle\psi_n^{(1)}(V - V_n)\psi_n^{(1)}\rangle - 2E_n^{(2)}\langle\psi_n^{(1)}\psi_n^{(0)}\rangle. \tag{5}$$

The expectation value of an operator $L$ in the nth state of the perturbed system may be expanded into different orders:

$$\langle L\rangle_n = \langle\psi_n L \psi_n\rangle / \langle\psi_n\psi_n\rangle = L_n^{(0)} + L_n^{(1)} + \ldots, \tag{6}$$

where $$L_n^{(0)} = L_n = \langle\psi_n^{(0)} L \psi_n^{(0)}\rangle, \; L_n^{(1)} = 2\langle\psi_n^{(1)}(L - L_n)\psi_n^{(0)}\rangle. \tag{7}$$

It has been pointed out by Dalgarno that in cases where the complexity of the perturbation $V$ makes the first-order equation (3) too difficult to solve explicitly, it may nevertheless be possible to solve the first-order equation

$$(H_0 - E_n^{(0)})\chi_n + (L - L_n)\psi_n^{(0)} = 0. \tag{8}$$

The first-order correction (7) may then be written in the form

$$L_n^{(1)} = 2<\chi_n(V - V_n)\psi_n^{(0)}>. \tag{9}$$

The integral in (9) is usually tractable even if $V$ contains two-electron terms (such as $1/r_{12}$), so that the correction is obtainable if (8) can be solved, which may be possible if $L$ is a one-electron operator. The mathematical problem is therefore to solve one-electron first-order wave equations of the type (8).

*Reduction of the First-Order Wave Equation.*—The device used to simplify (8) is to put

$$\chi_n = f_n\phi_n, \tag{10}$$

where we have replaced $\psi_n^{(0)}$ by $\phi_n$ for convenience, and to note that $(H_0 - E_n^{(0)})f_n\phi_n = (H_0f_n - f_nH_0)\phi_n$, so that (8) may be written

$$[H_0,f_n]\phi_n + (L - L_n)\phi_n = 0, \tag{11}$$

where the square brackets denote a commutator. This means that if, as usual,

$$H_0 = -{}^1/_2\nabla^2 + U_0 \tag{12}$$

where the unperturbed potential energy $U_0$ is a scalar, then (11) becomes

$$[\nabla^2,f_n]\phi_n = 2(L - L_n)\phi_n. \tag{13}$$

If this equation is multiplied by $\phi_n$, which we shall assume to be real, it may be rearranged to give

$$\nabla\cdot(\phi_n^2\nabla f_n) = 2\phi_n(L - L_n)\phi_n, \tag{14}$$

which is equivalent to the Wronskian form

$$\nabla\cdot(\phi_n\nabla\chi_n - \chi_n\nabla\phi_n) = 2\phi_n(L - L_n)\phi_n. \tag{15}$$

Since the usual boundary conditions are that $\phi_n$ and $\chi_n$ vanish at the boundaries, the boundary condition on $f_n$ is that

$$\phi_n^2\nabla f_n = 0 \text{ on boundaries.} \tag{16}$$

*Solution of the First-Order Wave Equation for One Dimension.*—For a one-dimensional system described by a variable $x$ confined to the interval $(a,b)$, equation (14) becomes

$$\frac{d}{dx}\left(\phi_n^2\frac{df}{dx}\right) = 2\phi_n(L - L_n)\phi_n. \tag{17}$$

This may be integrated directly to give

$$\phi_n^2\frac{df_n}{dx} = M_n(x) = 2\int_a^x \phi_n(\xi)(L - L_n)\phi_n(\xi)d\xi, \tag{18}$$

and the constant of integration vanishes because of the boundary condition (16).

The same treatment may be applied to a three-dimensional system described by spherical coordinates in which the operator $L$ depends only on $r$, and the unperturbed wave function is of the form $\phi_n(r,\theta,\varphi) = R_n(r)\Theta_n(\theta,\varphi)$. In this case $f_n$ depends only on $r$, and the equation (14) reduces to

$$\frac{d}{dr}\left(r^2R_n^2\frac{df_n}{dr}\right) = 2r^2R_n(L - L_n)R_n, \tag{19}$$

which is of the same form as (17) and may be integrated directly from the lower limit $r = 0$ to give

$$r^2R_n^2\frac{df_n}{dr} = P_n(r) = 2\int_0^r s^2R_n(s)(L - L_n)R_n(s)\,ds. \tag{20}$$

(a) *Ground state:* For the ground state, say $n = 0$, in which the unperturbed wave function $\phi_0(x)$ does not possess any nodes between $a$ and $b$, (18) may be integrated immediately to give

$$f_0(x) = f_0(a) + \int_a^x \frac{M_0(\xi)}{\phi_0^2(\xi)}\,d\xi, \tag{21}$$

as pointed out by Young and March. It should be noticed that this integration is not required explicitly if $\chi_0 = f_0\phi_0$ is to be used in an integral such as (9). This is because $L_0^{(1)} = 2\int_a^b f_0\phi_0(V - V_0)\phi_0 dx$ may be transformed by partial integration to give

$$L_0^{(1)} = -\int_a^b \frac{df_0}{dx}W_0(x)dx = -\int_a^b \frac{M_0(x)W_0(x)}{\phi_0^2(x)}\,dx, \tag{22}$$

where

$$W_n(x) = 2\int_a^x \phi_n(\xi)(V - V_n)\phi_n(\xi)d\xi. \tag{23}$$

In particular the second-order energy becomes

$$E_0^{(2)} = -{}^1/_2\int_a^b [W_0(x)/\phi_0(x)]^2dx. \tag{24}$$

The only point requiring special discussion in the radial case when $R_n(r)$ has no nodes is the behavior of $f_n(r)$ at the lower limit $r = 0$. If $R_n(0) \neq 0$, then $f_n(0)$ is finite as long as $LR_n(r)$ does not go to infinity faster than $r^{-1}$ at the origin.[3] If $R_n(r) \simeq r^l$ at the origin, then $f_n(0)$ is finite as long as $LR_n(r)$ goes to zero as fast as $r^{l-1}$ at the origin. The formula corresponding to (22) in this case is

$$L_n^{(1)} = -\int_0^\infty \frac{P_n(r)Q_n(r)}{r^2R_n^2(r)}\,dr \tag{25}$$

where

$$Q_n(r) = 2\int_0^r s^2R_n(s)(V - V_n)R_n(s)ds. \tag{26}$$

(b) *Excited states:* Consider the case in which the nth unperturbed eigenfunction $\phi_n(x) = \psi_n^{(0)}$ has $n$ simple zeros at $a_1, a_2, \ldots, a_n$ in the interval $(a,b)$. Then if the first-order wave function $\chi_n = \psi_n^{(1)}$ is well-behaved, the function $f_n(x)$ will have simple poles at $a_1, \ldots, a_n$. The direct integration of (18) to yield $f_n$ presents difficulties because $M_n(x)$ does not vanish in general at the zeros $a_j$. Similarly the integral for $L_n^{(1)}$ corresponding to (22), namely,

$$L_n^{(1)} = -\int_a^b \frac{M_n(x)W_n(x)}{\phi_n^2(x)}\,dx, \tag{27}$$

is infinite if $\phi_n$ vanishes in the range $(a,b)$.

To avoid the difficulties raised by the singularities in (18) and (27), let us assume

that $\phi_n$, $f_n$, $M_n$, and $W_n$ are analytic functions in the vicinity of the real interval $(a,b)$. Then equation (18) may be written

$$\frac{df_n(z)}{dz} = \frac{M_n(z)}{\phi_n^2(z)} \tag{28}$$

and integrated from $a$ to $x$ along any contour $C$ which does not pass through the zeros of $\phi_n$:

$$f_n(x) = f_n(a) + \oint_a^x \frac{M_n(z)}{\phi_n^2(z)}\, dz. \tag{29}$$

By Cauchy's theorem, $C$ may be any of the contours connecting $a$ and $x$ shown in Figure 1, since the integrand $M_n/\phi_n^2$ in (29) always has *double* poles at the points $a_1, \ldots, a_n$, and these do not contribute any residues to a contour enclosing them.

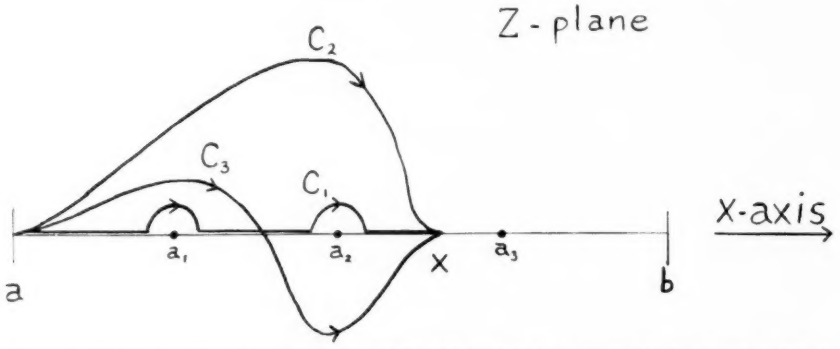


FIG. 1.—Three possible contours, $C_1$, $C_2$, and $C_3$, which might be traversed in the complex plane in the integration between the limit point $a$ and an arbitrary point $x$.

The nature of the poles of $M_n/\phi_n^2$ follows from the simple character of the zeros of $\phi_n$ and the definition of $M_n$ by equation (18), according to which $M'_n(a_j) = 0$, and therefore, in the vicinity of $a_j$, $M_n(z) = M_n(a_j) + 0(z - a_j)^2$. The same considerations apply to the integral expression for $L_n^{(1)}$ in equation (27), and the correct form is

$$L_n^{(1)} = -\oint_a^b \frac{M_n(z)W_n(z)}{\phi_n^2(z)}\, dz, \tag{30}$$

where the path of integration $C$ from $a$ to $b$ avoids the double poles at $a_1, \ldots, a_n$.

The radial case can be dealt with in the same way, and leads to the formula

$$L_n^{(1)} = -\oint_0^\infty \frac{P_n(z)Q_n(z)}{z^2R_n^2(z)}\, dz. \tag{31}$$

It follows that when the integrations in (30) or (31) can be performed analytically it is unnecessary to consider the singularities explicitly, since formally it makes no difference whether one integrates along a contour with complex variable $z$ or along the real axis with variable $x$. To clarify the method and to illustrate the power of

the integral formulae for excited states, it is useful to treat a simple example, for which we choose the polarizability of a particle in a one-dimensional box.

(c) *Example:* Consider a charged particle of unit mass whose position is described by $x$ and is free to move in the interval $(0,\pi)$. The eigenfunctions and energies are $\phi_n(x) = \sqrt{2/\pi}\sin(nx)$, $E_n^{(0)} = {}^1/_2 n^2$, $(n = 1,2,\ldots)$. Now suppose a perturbation $V = x - \pi/2$ is applied, corresponding to the energy of the particle in an electric field. The polarizability is $-2E_n^{(2)}$ where $E_n^{(2)}$ is the second-order energy of the nth eigenstate given by an integral similar to (30), namely,

$$E_n^{(2)} = -{}^1/_2 \oint_0^\pi [W_n(z)/\phi_n(z)]^2 dz \tag{32}$$

where the contour $C$ from 0 to $\pi$ avoids the singularities of the integrand at $k\pi/n$, $(k = 1,2,\ldots,n-1)$. The function $W_n(z)$ is given by

$$W_n(z) = \frac{1}{\pi}\left[z(z-\pi) - \left(z - \frac{\pi}{2}\right)\frac{\sin(2nz)}{n} + \frac{\sin^2(nz)}{n^2}\right]. \tag{33}$$

When this expression is substituted into (32) and the integration performed analytically ignoring the singularities, we obtain finally

$$E_n^{(2)} = -\frac{1}{24n^2}\left(\frac{15}{n^2} - \pi^2\right). \tag{34}$$

This agrees with the result obtained after much algebraic labor by summing the usual spectral formula

$$E_n^{(2)} = \sum_{\substack{k=1 \\ (\neq n)}}^{\infty} \frac{V_{nk}^2}{E_n^{(0)} - E_k^{(0)}}. \tag{35}$$

Note that it follows from (35) that $\sum_{n=1}^{\infty} E_n^{(2)} = 0$, which can be used to check (34).

*Removal of Singularities.*—When the integration is to be carried out numerically by a quadrature designed for integration along the real axis, it is necessary to devise ways of removing the singularities. There are two simple and apparently different ways of doing this, which are, however, related to each other.

(a) *Removal of singularities by subtraction:* The function $f_n$ defined by (10) has $n$ simple poles at $a_1, \ldots, a_n$ which may be revealed explicitly by defining a new function $g_n(x)$ by

$$f_n = g_n - \sum_{j=1}^{n} \frac{c_j}{x - a_j} \tag{36}$$

and choosing the $c_j$ so that $g_n$ is analytic at the points $a_j$. By substituting (36) into the differential equation (18) we get

$$\frac{dg_n}{dx} = \frac{M_n(x)}{\phi_n^2(x)} - \sum_{j=1}^{n} \frac{c_j}{(x - a_j)^2}. \tag{37}$$

The coefficients $c_1, \ldots, c_n$ must therefore be chosen to remove the singularities of $M_n/\phi_n^2$; that is,

$$c_j = M_n(a_j)/[\phi_n'(a_j)]^2, \ (j = 1,2,\ldots,n). \tag{38}$$

Equation (37) may now be integrated along the real $x$-axis to give

$$g_n(x) = g_n(a) + \int_a^x \left[\frac{M_n(\xi) - \Lambda_n(\xi)}{\phi_n^2(\xi)}\right] d\xi, \tag{39}$$

where

$$\Lambda_n(x) = \phi_n^2(x) \sum_{j=1}^{n} \frac{c_j}{(x - a_j)^2}. \tag{40}$$

Similarly equation (30) may be written as an integral along the real axis

$$L_n^{(1)} = -\int_a^b W_n \left[\frac{M_n - \Lambda_n}{\phi_n^2}\right] dx - \sum_{j=1}^{n} c_j \int_a^b \frac{\phi_n(V - V_n)\phi_n}{(x - a_j)}\, dx. \tag{41}$$

In the radial case, equation (36) is still valid with the coefficients given by

$$c_j = P_n(a_j)/[a_j R_n'(a_j)]^2. \tag{42}$$

The equation corresponding to (31) then becomes

$$L_n^{(1)} = -\int_0^\infty Q_n \left[\frac{P_n - \Gamma_n}{r^2 R_n^2}\right] dr - \sum_{j=1}^{n} c_j \int_0^\infty \frac{r^2 R_n(V - V_n)R_n}{(r - a_j)}\, dr, \tag{43}$$

where

$$\Gamma_n(r) = r^2 R_n^2 \sum_{j=1}^{n} \frac{c_j}{(r - a_j)^2}. \tag{44}$$

(b) *Removal of singularities by lower states:* An alternative method is to make use of the $n$ eigenfunctions $\phi_0, \ldots, \phi_{n-1}$ for the lower states of the unperturbed system. In place of (10) we write the first-order wave function in the form

$$\chi_n = g_n\phi_n - \sum_{k=0}^{n-1} b_k\phi_k, \tag{45}$$

where the coefficients $b_0, \ldots, b_{n-1}$ are constants to be chosen so that $g_n$ is analytic at $a_1, \ldots, a_n$. By substituting (45) for $\chi_n$ into (8) and proceeding as before, we get in place of (17)

$$\frac{d}{dx}\left(\phi_n^2 \frac{dg_n}{dx}\right) = 2\phi_n(L - L_n)\phi_n - 2\sum_{k=0}^{n-1} b_k(E_k - E_n)\phi_n\phi_k, \tag{46}$$

which on integration gives

$$\phi_n^2 \frac{dg_n}{dx} = M_n(x) - 2\sum_{k=0}^{n-1} b_k(E_k - E_n)\int_a^x \phi_n\phi_k\, d\xi. \tag{47}$$

The second integral may be evaluated immediately by means of the unperturbed Schrödinger equations satisfied by $\phi_k$ and $\phi_n$, namely,

$$\frac{d^2\phi_k}{dx^2} + 2(E_k - U_0)\phi_k = 0, \quad \frac{d^2\phi_n}{dx^2} + 2(E_n - U_0)\phi_n = 0. \tag{48}$$

By multiplying by $\phi_n$ and $\phi_k$ respectively, and subtracting, we get

$$\frac{d}{dx}\left(\phi_k \frac{d\phi_n}{dx} - \phi_n \frac{d\phi_k}{dx}\right) = 2(E_k - E_n)\,\phi_k\phi_n, \tag{49}$$

and hence by integration

$$\phi_k\phi_n' - \phi_n\phi_k' = 2(E_k - E_n)\int_a^x \phi_k\phi_n\, d\xi. \tag{50}$$

When (50) is substituted into (47), the latter becomes

$$\phi_n^2 \frac{dg_n}{dx} = M_n - \sum_{k=0}^{n-1} b_k(\phi_k\phi_n' - \phi_n\phi_k'). \tag{51}$$

The $n$ coefficients $b_k$ have now to be chosen so that the two terms on the right-hand side cancel to the first-order in $(x - a_j)$ at the points $a_j$. This can be achieved if the $b_k$ satisfy the $n$ linear simultaneous equations

$$\sum_{k=0}^{n-1} b_k\phi_k(a_j) = M_n(a_j)/\phi_n'(a_j), \ (j = 1, \ldots, n). \tag{52}$$

It follows directly from (49) that the derivative of the right-hand side vanishes automatically at the points $a_j$, so that (51) may be integrated along the real axis to give

$$g_n(x) = g_n(a) + \int_a^x \left[\frac{M_n(\xi) - \Omega_n(\xi)}{\phi_n^2(\xi)}\right] d\xi, \tag{53}$$

where

$$\Omega_n = \sum_{k=0}^{n-1} b_k(\phi_k\phi_n' - \phi_n\phi_k'). \tag{54}$$

The corresponding formula for $L_n^{(1)}$ is then

$$L_n^{(1)} = -\int_a^b W_n\left[\frac{M_n - \Omega_n}{\phi_n^2}\right] dx - \sum_{k=0}^{n-1} b_k V_{kn}, \tag{55}$$

where

$$V_{kn} = \int_a^b \phi_k V \phi_n\, dx. \tag{56}$$

This form would not be of interest unless it was possible to solve (52) explicitly for the $b_k$ in a large class of cases. The class is that in which the unperturbed functions $\phi_k$ form the basis for a Gauss-type quadrature formula with weight factor $\phi_0^2(x)$. In this case, quadrature weights $A_j$ may be found so that

$$\int_a^b \phi_0^2(x)p(x)dx = \sum_{j=1}^{n} A_j\phi_0^2(a_j)p(a_j) \tag{57}$$

exactly for all polynomials $p(x)$ up to degree $2n - 1$. The $A_j$ are given by

$$A_j = \left[\sum_{k=0}^{n-1} \phi_k^2(a_j)\right]^{-1} \tag{58}$$

or alternative formulae.[4] Applying equation (57) to $p = \phi_k\phi_n/\phi_0^2$, we get

$$\sum_{j=1}^{n} A_j\phi_k(a_j)\phi_l(a_j) = \delta_{kl}, \ (k,l = 0,1, \ldots, n - 1). \tag{59}$$

Therefore by multiplying (52) by $A_j\phi_k(a_j)$ and summing over $j$, we get the explicit form

$$b_k = \sum_{j=1}^{n} A_j\phi_k(a_j)M_n(a_j)/\phi_n'(a_j). \tag{60}$$

The class of functions $\phi_k$ to which this treatment applies is that in which $\phi_k$ is equal to $\phi_0$ times a polynomial of degree $k$ in $x$ (or, more generally, in a transformed variable). Thus it includes the Hermite functions for the simple harmonic oscillator but excludes the Laguerre function solutions of the hydrogen atom.

It will now be shown that in the cases in which this method can be applied, it is equivalent to the direct subtraction of the singularities. Consider the expansion of the function $\phi_n(x)/(x - a_j)$ in terms of the complete set of unperturbed functions $\phi_k$. For the class under consideration only the $n$ lowest functions $\phi_0, \ldots, \phi_{n-1}$ are involved so that

$$\phi_n(x)/(x - a_j) = \sum_{k=0}^{n-1} B_k \phi_k(x), \tag{61}$$

where $B_k = \int_b^a \frac{\phi_k \phi_n}{x - a_j}\, dx$. Now the quadrature formula (57) is exact in this case so that $B_k = A_j \phi_k(a_j) \phi_n'(a_j)$, and therefore

$$\frac{\phi_n(x)}{x - a_j} = A_j \phi_n'(a_j) \sum_{k=0}^{n-1} \phi_k(a_j) \phi_k(x). \tag{62}$$

Hence from (36) and (38) we have

$$\phi_n g_n - \chi_n = \phi_n \sum_{j=1}^{n} \frac{c_j}{x - a_j} = \sum_{j=1}^{n} c_j A_j \phi_n'(a_j) \sum_{k=0}^{n-1} \phi_k(a_j) \phi_k(x) = \sum_{k=0}^{n-1} b_k' \phi_k(x), \tag{63}$$

where $$b_k' = \sum_{j=1}^{n} c_j A_j \phi_n'(a_j) \phi_k(a_j) = \sum_{j=1}^{n} A_j M_n(a_j) \phi_k(a_j)/\phi_n'(a_j) = b_k \tag{64}$$

by equations (30) and (60). Thus (63) is the same as (45), which proves that in the restricted class of cases under consideration, the two methods are identical; the same arguments apply to the radial equation. While the second method is theoretically interesting in suggesting that quadrature formulae may be useful in perturbation theory, the first method is quite general and is naturally to be preferred in numerical applications.

The authors wish to thank J. B. McLeod (Wadham College, Oxford) for suggesting the analytical method of avoiding the singularities.

* This research was supported jointly by the following contract and grant: U.S. Air Force contract AF 33(657)-7311, and National Aeronautics and Space Administration grant NsG-275-62.

[1] Dalgarno, A., *Quantum Theory*, ed. D. R. Bates (New York: Academic Press, 1961), vol. 1, chap. 5; Dalgarno, A., "Advances in Physics," *Phil. Mag. Supplement*, **11**, 281 (1962); Schwartz, C., *Ann. Phys.*, **2**, 156, 170, and 176 (1959).

[2] Young, W. H., and N. H. March, *Phys. Rev.*, **109**, 1854 (1958).

[3] If $LR_n(r)$ goes to infinity even as fast as $r^{-2}$ at the origin, no serious difficulties are encountered. However, in this case $f_n(0)$ might be infinite. For example, if $LR_n(r)$ goes to infinity as $r^{-2}$ at the origin, then $f_n(0)$ contains a term $A \ln(r)$.

[4] Kopal, Z., *Numerical Analysis* (London: Chapman and Hall, 1955), chap. 7.

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